การแยกและวิเคราะห์ยีนที่ควบคุมขบวนการ apoptosis ในพยาธิเท้าช้าง

บทคัดย่อ

ของ

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โรคเท้าช้างเป็นโรคที่เป็นปัญหาสำคัญทางสาธารณสุขในเขตร้อนและบริเวณเขตร้อนชื้น รวมทั้งในประเทศไทย องค์การอนามัยโลก (WHO) ได้ตั้งเป้าหมายที่จะกำจัดโรคเท้าช้างให้ได้ภายในปี 2020 สาเหตุของโรคมาจากพยาธิ *Wuchereria bancrofti, Brugia malayi* และ *B. timori* ซึ่งพยาธิ *B. malayi* สามารถพบได้มากในเขตภาคใต้ของประเทศไทยและเป็นสาเหตุของโรคเท้าช้างในคนโดยมีแมวและยุงเป็นแหล่งสะสมเชื้อและพาหะของโรคตามลำดับ กลไก apoptosis เป็นกระบวนการที่คาดว่าเกี่ยวข้องกับการเจริญเติบโตของพยาธิ *B. malayi* ในระยะที่อยู่ในคนและยุง อย่างไรก็ตามกระบวนการ apoptosis ยังไม่มีการรายงานในพยาธิ *B. malayi* ดังนั้นการศึกษานี้จึงมุ่งเน้นไปที่ยีนของ apoptosis ในพยาธิ *B. malayi* โดยใช้ยีนของ *C. elegans* เป็นต้นแบบ

ในการวิจัยได้ทำการแยกยีนของ apoptosis จากพยาธิ *B. malayi* ด้วยเทคนิค PCR โดยใช้ไพรเมอร์ที่ออกแบบจากยีน *ced-3, ced-4* และ *ced-9* ของพยาธิ *C. elegans* ผลจากการเพิ่มขยายยีนโดยไพรเมอร์ *ced-3* ปรากฏว่าได้ยีนที่มีขนาด 569 bp ซึ่งมีลำดับเบสเหมือนกับจีโนมพยาธิ *B. malayi* และยีน *ced-3* ของพยาธิ *C. elegans* เท่ากับ 96% และ 52% ตามลำดับและจากการวิเคราะห์หา Open Reading Frame ปรากฏว่าไม่พบยีน คาดว่าลำดับเบสที่ได้นี้ อยู่ในส่วนของ intron ของยีน สำหรับไพรเมอร์ที่เพิ่มขยายยีนของ *ced-4* ปรากฏว่าได้ดีเอ็นเอขนาด 474 bp ซึ่งลำดับเบสมีความเหมือนกับจีโนมของ *B. malayi* และยีน *ced-4* ของ *C. elegans* เท่ากับ 92% และ 53% ตามลำดับ และพบส่วนที่เป็นยีนของโปรตีน แต่ไม่มีความเหมือนกับยีนใดๆ ใน GeneBank จากการแยกยีนโดยใช้ไพรเมอร์ที่ออกแบบมาจากยีน *ced-9* ได้ดีเอ็นเอที่มีลำดับเบสขนาด 742 bp ที่มีความเหมือนกับยีน rRNA ของพยาธิ *B. malayi* และ *ced-9* ของ *C.elegans* เท่ากับ 98% และ 46% ตามลำดับ

จากผลการทดลองแสดงให้เห็นว่า ส่วนของยีนที่ได้มาจากดีเอ็นเอของ *B. malayi* โดยที่ลำดับเบสมีความเหมือนกับยีน apoptosis ของ *C. elegans* ประมาณ 50% ดังนั้นในการศึกษาต่อไปน่าจะทำการแยกยีนทั้งหมด เพื่อทำการศึกษากระบวนการ apoptosis ใน *B. malayi* ซึ่งจะได้ข้อมูลใหม่และอาจนำไปใช้ในการออกแบบยารักษาโรคพยาธิเท้าช้างได้

ISOLATION AND ANALYSIS OF AN APOPTOTIC GENE IN LYMPHATIC FILARIAL NEMATODE

AN ABSTRACT

BY

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Lymphatic filariasis is a disease that has been the major public health problem in tropical and subtropical world including Thailand. The World Health Organization (WHO) has targeted eradication lymphatic filariasis in the year 2020. This disease is caused by *Wuchereria bancrofti, Brugia malayi* and *B. timori* parasites. *B. malayi* is mostly found in southern part of Thailand. It causes elephantiasis in human and has cat and mosquito as a reservoir and vector, respectively. Apoptosis pathway is a process that supposed to involve in the development of *B. malayi* in human and mosquito stages. However, this process has not been reported. Therefore, in this study, apoptotic genes of *B. malayi* were determined by using apoptotic genes of *C. elegans* as a model.

The putative apoptosis genes of *B. malayi* were isolated by PCR technique using primers that designed from *ced-3, ced-4* and *ced-9* genes of *C. elegan*s. The result of *ced-3* primer amplification gave a 569 bp DNA fragment that the nucleotide sequence was similar to *B. malayi* genome and *ced-3* gene of *C. elegan*s at 96% and 52%, respectively. The analysis of the open reading frame revealed that it was in the intron part of the gene. The amplification using *ced-4* primer gave a DNA fragment sized of 474 bp which the sequence similar to *B. malayi* and *ced-4* gene *of C. elegan*s at 92% and 53 %, respectively. However, the putative protein from this ORF showed no homology with any genes in the GenBank. The nucleotide sequence of 712 bp resulted from *ced-9* primers showed 98 % and 46% similarity to rRNA gene of *B. malayi* and *C. elegan*s *ced-9* gene, respectively.

From these results, it revealed that the amplification products were from *B. malayi* DNA. These partial nucleotide sequences were similar to apoptotic genes of *C. elegan*s about 50%. For further study, full-length genes isolation are needed for fully characterized apoptotic genes of *B. malayi* which will be a new data and may be used as drug target for filariasis treatment.

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CHAPTER 1

INTRODUCTION

Lymphatic filariasis, commonly known as elephantiasis, is caused by the thread-like parasitic filarial worm *Wuchereria bancrofti, Brugia malayi* and *B. timori.* This disease infects 140 million people in 80 countries world wide and continues to be a worsening problem, especially in tropical and subtropical world. In Thailand, the infection of *W. bancrofti* is found in northern and western, but *B. malayi* is found in the southern part.[1-8]

Elephantiasis have parasite in their blood and hidden internal damage to their lymphatic and renal systems. The disease has been the major public heath problem. The World Heath Organization (WHO) has targeted the year 2020 for eradication of lymphatic filariasis.[9-13]

The transmission of filarial parasites is via the bite of blood-feeding female mosquitoes; *Culex quinquefasciatus* (*W. bancrofti)* and *Mansonia mosquitoes* (*B. malayi*), which transmit immature larval forms from human to human. In humans, adult worms produce large numbers of larval forms (known as microfilariae) which circulate in the lymphatic and blood where they can be ingested by blood-feeding mosquitoes. Thereafter, it molts into the second stage (L2) and infective larva (L3). The L3 migrates through the mouth parts of the mosquito. Infection of the definitive host takes place when the mosquito takes a blood meal.[1-8]

The development of the worm to several stages in the life cycle may need program cell death or apoptosis process. Normally, apoptosis is one of the processes that play an important role in the development as cell differentiation or cell proliferation of multi-cellular organisms. It regulates the elimination of unnecessary and dangerous cell in tissues.[16-19]

Four genes making up the core apoptosis pathway in *Caenorhabditis elegans*, three of which, *egl-1* (egg laying abnormal), *ced- 3* (cell death abnormal), and *ced-4* are pro-apoptotic and *ced- 9*, is anti-apoptotic. There was considerable excitement in the field when potential mammalian and *Drosophila* homolog for *ced-3, ced-4, ced-9* and *egl-1* were discovered.[24-41]

Apoptosis can be initiated by multiple internal and external triggers and coordinated by a complex network of regulators and effectors. The various triggers include factors such

as cellular stress, serum or growth factor deprivation, receptor ligands, de-regulation of cell division, and development or differentiation.

In this study, we interested in apoptosis genes of *B. malayi* for better understanding the apoptosis mechanism in this parasite, which leads to a new drug targets for treatment of *B. malayi* in the future. The gene in *C. elegans* was used as a model for gene isolation because it was close-related organism and well studied.

CHAPTER 2

LITERATURE REVIEW

1. Lymphatic filarial nematode

Lymphatic filariasis is caused primarily by adult worms (filariae) that live in the lymphatic vessels. The female worms release microfilariae that circulate in the peripheral blood and are ingested by mosquitoes; thus, infected mosquitoes transmit the infection from person to person. The two major species of filariae that cause lymphatic disease in humans are *Wuchereria bancrofti*, *Brugia malayi* (Fig. 1 A and 1 B) and *B. timori*.[1-5]

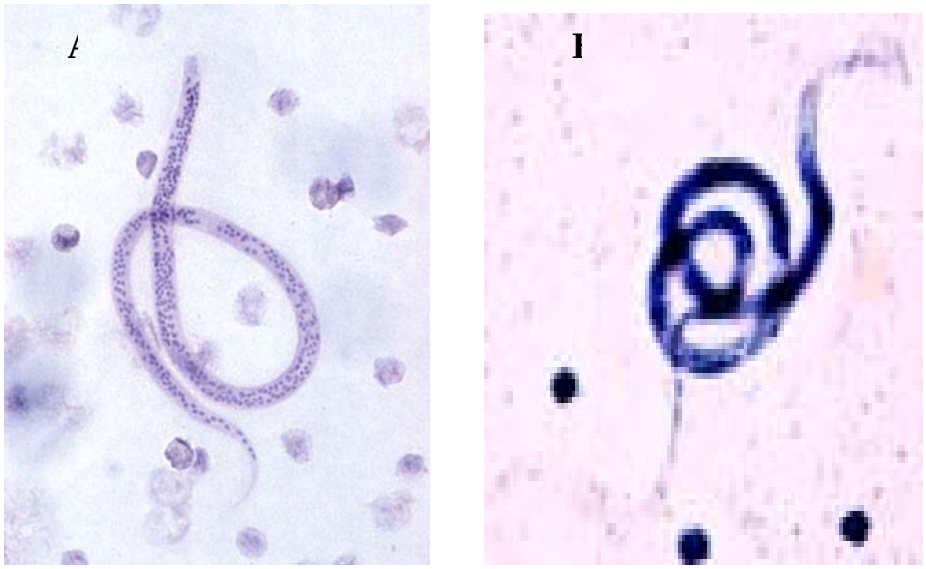


Figure 1 Morphology of *Wuchereria bancrofti* (A) and *Brugia malayi* (B)[6]

Filarial nematodes infect human lymphatic system, subcutaneous and deep connective tissue resulted in filariasis diseases. The most important of the lymphatic filariasis is known as elephantiasis. In this case, adult worm cause the damage to the lymphatic vale system which cause the lymph to drain to the extremities invading and swelling the subcutaneous tissue. Elephantiasis has been written since the time of the early Greek and Romans.[3-4]

Most of filarial infections are asymptomatic, but the living adult worm causes progressive lymphatic vessel dilation and dysfunction. Lymphatic dysfunction leads to lymphedema of the leg (Fig. 2A), scrotum (Fig. 2B), penis, arm, or breast, which can

increase in severity as a result of recurrent secondary bacterial infections. Tropical pulmonary eosinophilia is a potentially serious progressive lung disease with nocturnal cough, wheezing, and fever, resulting from immune hyper-responsiveness to microfilaria in the pulmonary capillaries.[2-4]

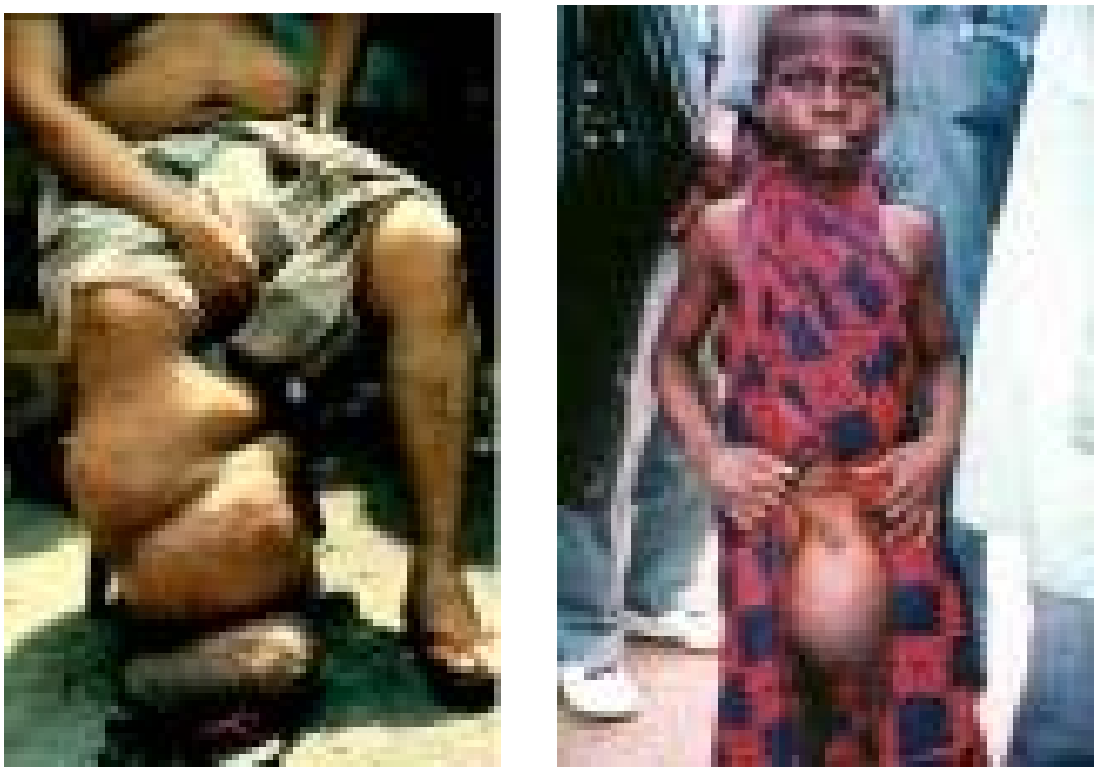


Figure 2 Lymphatic dysfunction leads to lymphedema of the leg (A) and scrotum (B)[7]

Lymphatic filariasis affects an estimated 140 million persons in tropical areas of the world, including sub-Saharan Africa, Egypt, southern Asia, the western Pacific islands, the northeastern coasts of South and Central America, and the Caribbean Islands.[8-10]

1.1 Taxonomy

Human filariasis is classified as indicated below.[8]

Phylum Nematoda

Class Secernentea

Subclass Spiruria

Order Spirurida

Suborder Spirurina

Superfamily Filarioidea

Family Filariidae

Genus *Wuchereria* sp., *Brugia* sp.

Species *Wuchereria bancrofti, Brugia malayi*

1.2 Distribution

Endemic in over 80 countries in Africa, Asia, South and Central America and the Pacific Islands. More than 40% of all infected people live in India and one-third live in Africa (Fig.3).[8-10]

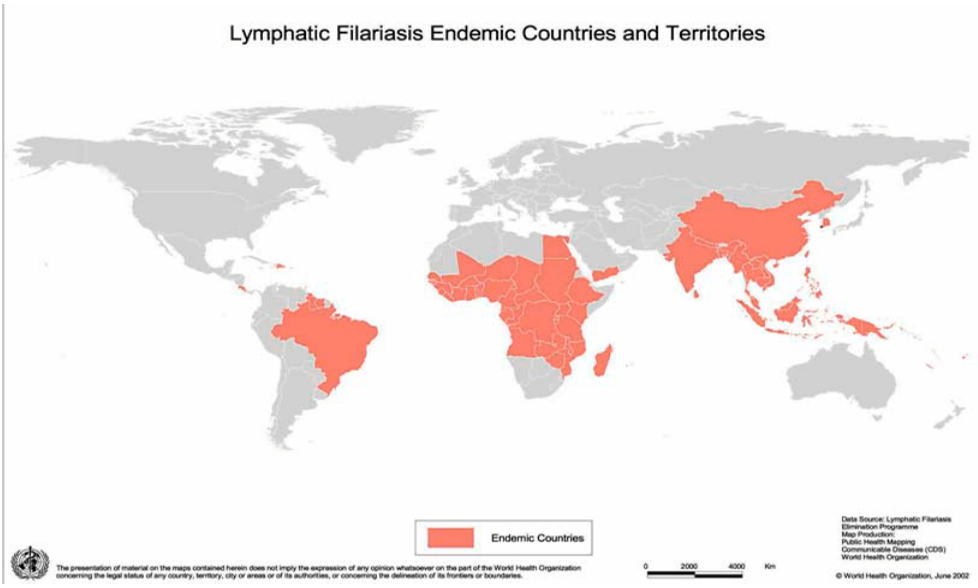


Figure 3 The global distribution of lymphatic filariasis.[11]

In Thailand, the infection of *W. bancrofti* is found in the northern and western part such as Mae Hongsorn, Tak, Kanchanaburi. For *B. malayi* is mostly found in the southern part such as Narathiwat, Surajthani, Yala.[5] (Fig. 4)

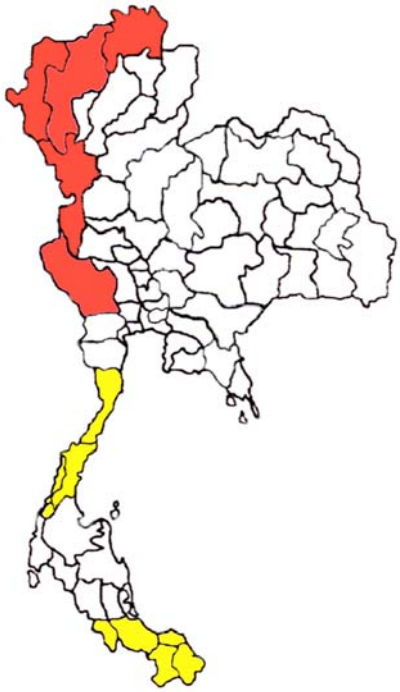


Figure 4 The distribution of lymphatic filariasis in Thailand.

1.3 Morphology

Adult worms of *W. bancrofti* are long and slender with a smooth cuticle and bluntly rounded end. The head is slightly swollen and bears two circles of well–defined papillae. The mouth is small, a buccal cavity is lacking. The males are smaller than the females. The male is about 40 mm x 100 μm. Its tail is finger-like. The female is 6-10 cm x 300 μm. The vulva is near the level of the middle of the esophagus. Adult worm of *B. malayi* is very similar to that of *W. bancrofti*, although it is only about half as large. The male are 13.5 – 20.5 mm x 70-80 μm. Its tail is curved ventrad and bears three or four pairs of adrenal and three or four pairs of postanal papillae. The spicules are unequal and dissimilar, and a small gubernaculum is present. The females are 8-100 mm x 240-300 μm. The finger-like tail is covered with minute cuticular bosses. The vulva is near the level of the middle of the

esophagus, when mature produce worm-like sheathed egg or microfilariae which circulate, in blood.[1-3]

The microfilariae are easily seen in blood films and readily available for diagnosis. In blood, the length of microfilaria of *W. bancrofti* is about 245-300 μm and *B. malayi* is 200-275 μm (Fig. 1).[1, 3]

In *W. bancrofti*, the tail is no nuclei whereas *B. malayi* have terminal nuclei, body nuclei, extend thought-out the tail. The cephalic space canterior area free of nuclei, has a length–to–width ratio of 1.1 in *W. bancrofti*, but 2 :1 in *B. malayi*.[1]

1.4 Transmission

The transmission of filarial parasites is via the bite of blood-feeding female mosquitoes which transmit immature larval forms of the parasitic worms from human to human. *W. bancrofti* parasites are mainly transmitted by *Culex quinquefasciatus* mosquitoes and some species of Anopheles. Brugia parasites are mainly transmitted by *Mansonia mosquitoes*. In humans, adult worms can live for many years, producing large numbers of larval forms (known as microfilariae) which circulate in the lymphatics and blood where they can be ingested by blood-feeding mosquitoes, so completing the transmission cycle.[3, 8-10]

1.5 Life cycle

The adult worms are located in lymph vessels near lymph nodes. Adult females produce sheathed microfilariae that migrate into the lymph and blood vessel. The intermediate host (mosquitoes) becomes infected when they take a blood meal from infected human host. The microfilaria ingested by the mosquito pass through stomach where they lose the sheath after about 2 h. The larva penetrates the gut wall and migrates to the thoracic muscles, where after about 2 day, it molts into the second stage (L2) which has a short sausage shaped worm. After 2 week the larva molts form infective larva (L3), a slender filariform larva (1.4 –2 mm long). The L3 migrates through the haemocoel of the insect to reside in the mouth parts of the musquito at the labium which form the sheath for the proboscis. Infection of the definitive host takes place when the mosquito takes a blood meal. The route of the infecting juveniles is not completely known, but they apparently migrate in lymph vessels where they mature and finally locate in the lymph glands of the groin and lower extremities.

In most part of the world where filariasis is endemic, the infection of microfilariae present in very small numbers in the circulating blood during the daytime and often virtually undetectable. The maximal number of microfilariae usually found between 10 PM and 2

AM which call Periodic form. But, in the Pacific area, microfilaria increase in circulating blood between noon and evening which is called Subperiodic form.(2-3,7-8)

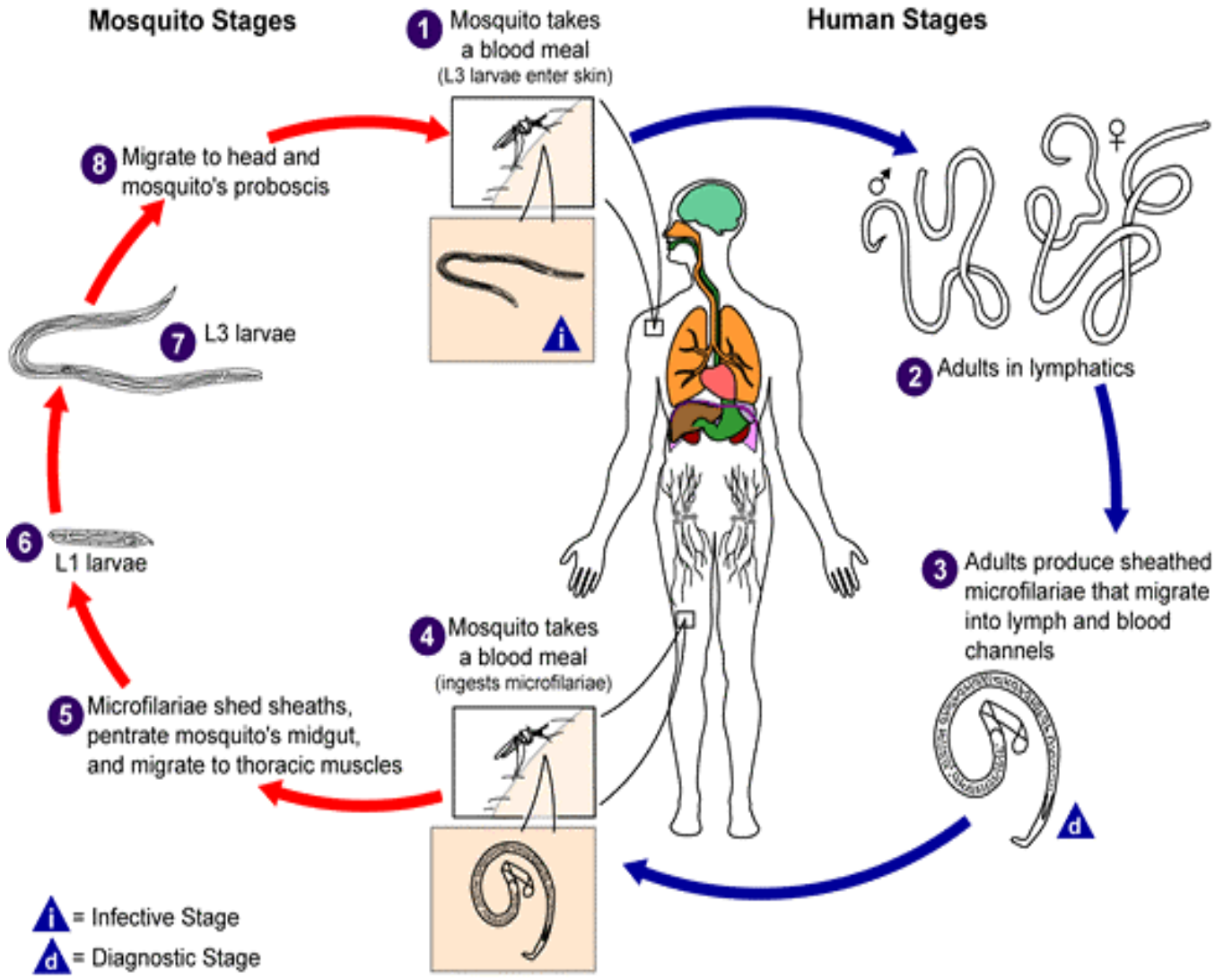


Figure 5 Life cycle of *Wuchereria* sp. and *Brugia sp*.[12]

1.6 Pathogenesis

Pathogenesis associated with lymphatic filariasis results from a complex interplay of the pathogenic potential of the parasite, the immune response of the host to the adult worm, particularly the female microfilaria, and external bacterial and fungal infections.

Filariasis can be regarded as 3 clinical phases: the incubation, acute or inflammatory and obstructive phase. In incubating phase, the infective larvae reach the lymphatic vessels within 2 to 3 days, where they grow and molt.

Of all the patients with lymphatic filariasis at least half appear clinically asymptomatic, though they have microfilariae circulating in their blood and essentially all have hidden damage to their lymphatic and/or renal systems. It is clear that this state of asymptomatic microfilaraemia is associated with a highly down-regulated immune system, but it is as yet unclear now, when or even whether these individuals will progress to develop one of the more overt clinical manifestations of filarial disease.

The acute inflammatory stage followed when the adult females are releasing large number of microfilariae. There is intense lymphatic inflammation accompanying with chills, fever and toxaemia. The swollen of lymph node can be very painful and the overlying skin may be reddene. The attack usually subsides after a few days. But this and the other manifestations described further often recur at frequent intervals at the cellular level which there is inflammatory cell infiltration of the lymph node linings, particularly polymorphonuclear, eosinophils and lymphocytes. Adult worms are frequently killed with abscesses forced around them. Microfilariae may disappear from the peripheral blood during and after the acute phase presumably because of the lymphatic vessel containing the female becomes blocked.

The obstructive phase is progress by the blockage of the lymphatic ducts and causes the backing of the lymphatic fluid which then passes into the surrounding tissue as a result of lymphedema. For chronic lymphedema, there is a hyperplasia of the connective tissue and infiltration of plasma cell, macrophages, and eosinophils, finally woody induration of the tissues may take place with thickening and verrucous changes of the skin producing the condition known as elephantiasis.[1-3,16-17]

1.7 Diagnosis of filariasis

Definitive diagnosis is limited by finding of microfilariae in blood. Giemsa stained of thick blood film is a commonly used. Polymerase chain reaction (PCR) based assays are available for the presence of *W. bancrofti* and *B. malayi* in blood and sputum. ELISA test for serum are also available and highly accurate. Detection of adult worms is usually difficult because they are deeply live within the lymphatic system, but may be detected by ultrasound of scrotum.[1-3]

1.8 Treatment

Ivermectin in a single oral dose of 100-400 mg/kg and sometime combined with albendozole. Alternative treatment (first-line in some references) is diethylcarbamazine (DEC) give in graduated doses as follows: 50 mg on the first day, 50 mg 3 times daily on day 2, l00 mg 3 times daily on day 3 and 2 mg/kg for 3 times daily thereafter. Annual single dose treatments with DEC at 6 mg/kg may be used in areas of high endemicity.[18-19]

While it is important to try to cure the infection itself, management of the consequences of that infection (particularly the lymphedema, elephantiasis and hydrocoeles) is what is often of greatest concern to the patient. Interestingly, with respect to hydrocoeles it has now been shown repeatedly that treatment of infection in communities with either intermittent (monthly, 6-monthly, yearly) drug administration or the steady use of DEC-fortified table/cooking salt, leads to clinical improvement with decreases in both hydrocoele size and prevalence. Similarly, it is not uncommon to find early lymphoedema regressing completely after treatment of an affected patient with DEC.[18-19]

In more chronic states, those larger hydrocoeles that do not regress spontaneously or after treatment must be subjected to surgical procedures in order to drain the fluid and render the tunica vaginalis incapable of trapping and retaining it again.

2. Apoptosis

Apoptosis or Programmed Cell Death (PCD) is of Greek original, having the meaning falling off or dropping. Programmed cell death was introduced in 1964, proposing that cell death during development follows a sequence of controlled step but of accidental nature. Later, apoptosis was first introduced in a publication in 1972 by Kerr, Wyllie and Currie,[20] they described the morphological processes leading to controlled cellular self – destruction.

Apoptosis is one of the processes that play an important role in the development as cell differentiation or cell proliferation of multicellular organisms. It regulates the elimination of unnecessary and dangerous cell in tissues.[21] Abnormal apoptosis regulation leads to variety diseases.[22] For example, excessive apoptosis can result in neurodegenerative disorders, AIDS, ischaemic, while defective is linked to cancer, autoimmune diseases and viral infections.[23]

3. Apoptosis and necrosis

Apoptosis and necrosis are two major processes by which cause cell die. Apoptosis is a form of program cell death that is characterized by specific morphological and biochemical properties.[24] Characterized morphological of apoptotic cell is structural changes in dying cell, cell shrinkage, blebbing of the plasma membrane, chromatin condensation and marginates at the nuclear membrane and cellular fragmentation into

compact membrane – enclosed structures which is called apoptotic bodies.[21,24] The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response. (Fig 6) Characterized biochemical, apoptosis is degraded of chromatin by the activation of proteolysis enzyme, initially into large fragment of 50 – 300 Kb and subsequently into smaller fragments that are monomers and multimers of 200 bp.[25-26]

These characteristics of apoptosis is different from necrosis which cause the cells suffer a major insult, injured cell to swell and lysis, releasing the cytoplasmic materials into the cell's environment which resulting in damage of surrounding cells and stimulate an inflammatory response in the corresponding tissue.[21] (Fig. 6)

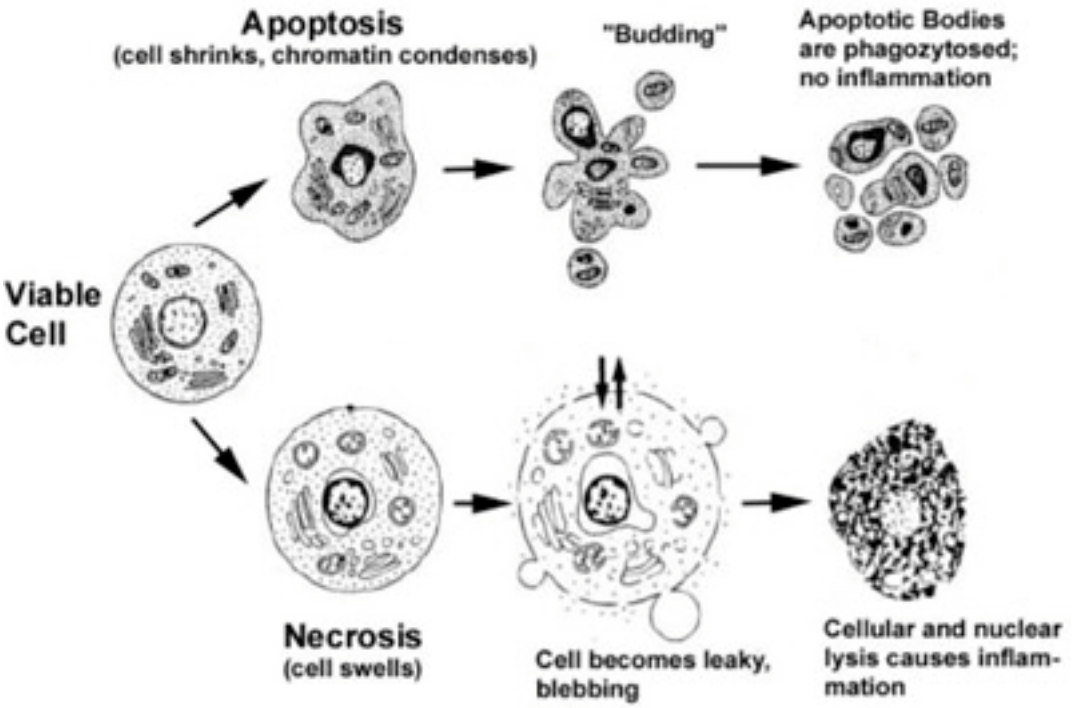


Figure 6 Morphological changes by apoptosis and necrosis process[27]

Apoptosis can be triggered by various stimuli from outside the cell, for example, ligands of cell surface receptors, cytotoxic drugs or irradiation. Apoptosis can also be triggered by stimuli inside the cell such as death signal, DNA damage etc.

4. *C. elegans* as a model of apoptosis process

The nematode *C. elegan*s has been the subject for intensive studies by Brenner in 1974,[28-29] who introduced this worm as an organism for genetic analysis. This nematode was chosen as a model because of the small size (its genome size was estimated to be 100 Mbp), rapid generation time, easily to growth and maintain in laboratory. Many researchers have adopted this nematode as their system of choice for the genetic dissection of basic biological processes. The advantage of studying cell death at the single-cell level in combination with the powerful genetics available in *C. elegans* has led to the identification of many mutation that affect the program in many aspects and also help to define genetic pathway that regulates and executes the Program.[30] A second advantage of studying cell death in *C. elegan*s is the availability of a detailed genetic map, the physical map of cosmid and yeast artificial chromosome (YAC) clones that cover the entire genome[31-32] and the almost completely sequence genome.[32]

5. Apoptosis in *C. elegans*

Since all cell division and their differentiation are the same among all *C. elegan*s, a cell lineage can be constructed for the series of cell changes that transform the fertilized egg into an adult. During development exactly 1,090 cells are formed, but only 959 of these cells comprise the adult *C. elegans*. Thus, 131 cells are eliminated by programmed cell death during development of *C. elegans.*[33-34]

Programmed cell death in *C. elegans* shares many morphological changes that are characteristic of apoptosis such as chromatin condensation and reduction in cell volume.[24] Cytosolic organells (eg. mitochondria) appear to be normal until very late of the process. At later stages, the dying cell breaks up into membrane-bound fragments, autophagic vacuoles and membranous appear in the cytoplasm. The engulfed cell fragments finally fuse with vacuoles, thus completing the elimination of the death cell.[35]

5.1 Genetics of apoptosis

Genetic studied have led to the identification of a large number of mutations that affect programmed cell death and are therefore thought to act in a central pathway of cell death.

Genetic pathway for programmed cell death contains four distinct, genetically separable steps: the decision of individual cell whether to live or to die, execution,

engulfment of the death cell and degradation of the engulfed cell. The gene act upstream of the general cell death pathway affect the decisions of small numbers of cell whether to live or to die, and are thought possibly to be involved in the cell type-specific control of the death program.[36-37]

5.2 Genes involve in apoptosis

Ced-3 and *ced-4* activate programmed cell death

The *ced-3* and *ced-4* genes are essential for programmed cell death in *C. elegans*. Mutations that inactivated of either gene resulted in the survival of 131 cells that normally undergo programmed cell death during *C. elegans* hermaphrodite development.[38] Programmed cell death in *C. elegans* is the activation of the CED-3 protein, which is mediation by its oligomerization at the activator protein CED-4. Since all of surviving cells with differentiate *ced-3* and *ced-4* mutant have 12% more somatic cells than their wild-type counterpath. *Ced-3* and *ced-4* mutants which affect the increasing of cell number does not affect on serious morphological or behavioral abnormalities but *ced-3* and *ced-4* mutant that defect the cell number slow growth and slightly reduced fertility, and are somewhat defective in complex tasks such as chemotaxis.[37]

The undead cells in *ced-3* and *ced-4* mutants never divide again, but rather differentiate, in most cases clearly adopting a cell fate very similar to the one adopted by their sister cell.[38] While the undead neurons show a greater variability in position and connectivity than their normal sister cells,[39] they are clearly healthy and normal-looking, suggestion that the absence of *ced-3* or *ced-4* function blocks the death program at an early stage, before any irreversible events have taken places.

Ced-3 gene sequence is homology to *ICE*- family in mammal

Cloning and characterization of the *ced-3* gene indicated that its product is a member of the interleukin-1β-converting enzyme (ICE) family of the cysteine proteases, suggesting that CED-3 might be a death–promoting protease, a hypothesis that has been supported by recent experimental findings.[40] The *C. elegans* cell death pathway is conserved through evolution, the sequence similarity between CED-3 and ICE also implied that members of the ICE family should be involved in mediating apoptosis in animals. Several other observations serve to support this prediction. First, overexpression of many different ICE family members can induce apoptosis. Second, an ICE-like protease activity can be detected in apoptotic, but not normal cells.[41-42] Third, and most importantly,

inhibition of ICE family protease prevents apoptosis,[42-43] indicating that, as in *C. elegans*, these proteases are not only sufficient but also necessary for the induction of apoptotic cell suicide.

Ced-9 inhibit in programmed cell death

The *ced-9* gene is a negative regulator of programmed cell death in *C. ele*gans.[44] Overexpression of *ced-9* resulted in the survival of cells that should be died and causes a phenotype similar to the one observed in animals that lack either *ced-3* or *ced-4*.[45] Survival of cells fated to die is also observed in mutants heterozygous or homozygous for a dominant gain-of-function mutation in the *ced-9* gene. Interestingly, this mutation does not result in overexpression of the wild-type protein, but rather result in a single amino acid change in a conserved domain of the CED-9 protein.[45]

By contrast, mutations that reduce or eliminate *ced-9* function cause many cells that normally live to inappropriately activate the cell death pathway and undergo programmed cell death. The widespread ectopic cell death in these mutants eventually results in the death of the animals (because of the death of essential cells). Thus, programmed cell death is not essential in *C. elegans* (because *ced-3* and *ced-4* mutants are viable and fertile), proper control of programmed cell death is clearly crucial even in this simple organism.

Ced-9 is homology to *Bcl-2* in mammals

The *ced-9* gene encodes a 280 amino acid protein with sequence and structural similarities to the mammalian *Bcl-2* gene.[46] Overexpression of *Bcl-2* in *C. elegans* under the control of a heat-shock promoter can prevent normal programmed cell death as well as the ectopic cell deaths that occur in *ced-9* loss-of-function mutants.[46-47] The amino acid sequence of *ced*-9 is 23% identical to that of *Bcl-2* . These results suggest that the function of *ced-9* and *Bcl-2* is evolutionarily conserved from *C. elegans* to mammals.

5.3 Genes involve engulfment and degradation of dying cell

Engulfment of cell undergoing programmed cell death is a highly efficient process in *C. elegans*; dying cells are phagocytosed and mostly digested within less than 1 h. Ultra-structural studies suggest that engulfing cell can recognize a dying cell before it shows any overt morphological changes and even before the cell division generation the dying cell has been completed.[35] Thus, generation of the engulfment-promoting signal must be an early event in the death process.

The group of six genes (*ced 1, 2, 5, 6, 7, 10*) involve in phagocytosis

The group of six genes (*ced 1, 2, 5, 6, 7, 10*) are required for this efficient phagocytosis. In animals, mutant for any one of these gene, cause the dying cells fail to be engulfed, and remain in the animal for hours, or even day.[48] The process in which these genes are involved appears to be partially redundant, as no single mutation completely blocks phagocytosis: even in the strongest mutants, over half of all dying cells are still engulfed with relatively normal kinetics.[38] Double mutant studies indicated that the six genes can be divided into two sets *(ced-l, 6, 7,* and *ced-2, 5, l0);* animals which mutant for one gene in each set show a much stronger defect than the animals which two genes mutant within the same class.[38] These observations suggest that *C. elegans* might possess two overlapping and partially redundant mechanisms that lead to the recognition and engulfment of apoptotic cells. It is interesting to note that at least three distinct mechanisms appear to be used to recognize apoptotic cells in mammals.[49] Whether the same mechanisms are used in *C. elegans* and mammals has not been determined.

Ced-8 involve in execution

Ced-8 had previously been classified as an engulfment-defective gene on the basis of the observation that, in *ced-8* mutants, threefold embryos show a significant higher number of cell corpses than the wild type.[38] However, *ced-8* mutants, both the appearance and engulfment of dying cell are slowed down, suggesting that *ced-8* is required for efficient and rapid execution of the death sentence rather than for engulfment per se.

Nuc-1 contain nuclease activity

The gene nuc-1 (nuclease deficient) is required to digest the DNA of dead, engulfed cell.[50] The *nuc-1* gene encoded nuclease involved in digestion of genomic DNA into nucleosome and scavenging endogenous DNA, and thus only has a peripheral role in programmed cell death

5.4 Apoptosis pathway in *C. elegans*

Three important apoptotic genes of *C. elegans* compose of *ced-3, ced-4* and *ced-9* which encode for ProCED-3, CED-4 and CED-9 proteins, respectively. The CED-4-dependent activation of CED-3 is important during apoptosis process and probably commits cell to the cell-death fate. The ability of CED-4 to activate CED-3 is blocked by the CED-9 protein, which directly interacts with CED-4. But, the function of CED-9 as anti-apoptosis is antagonized by the EGL-1 (egg-laying abnormal). The binding of EGL-1 to CED-9 results in the releasing of CED-4 from CED-9. Then, the CED-4 form a complex with CED-3 which activated CED-3 and induce execution phase of apoptosis. Three events result of CED-3

activation; (1) induction of morphological changes which are the characteristic of apoptotic cell; (2) eat-me signals expose on the cell surface and; (3) degradation of chromosomal DNA. The exposure of eat-me signals on apoptosis cell activates the engulfment machinery which composed *ced-l, 6, 7,* and *ced-2, 5, l0* and 12 genes in phagocytes. The engulfment genes *ced-1* and *ced-7* seem to be important in the degradation of chromosomal DNA in apoptotic cell.[51]

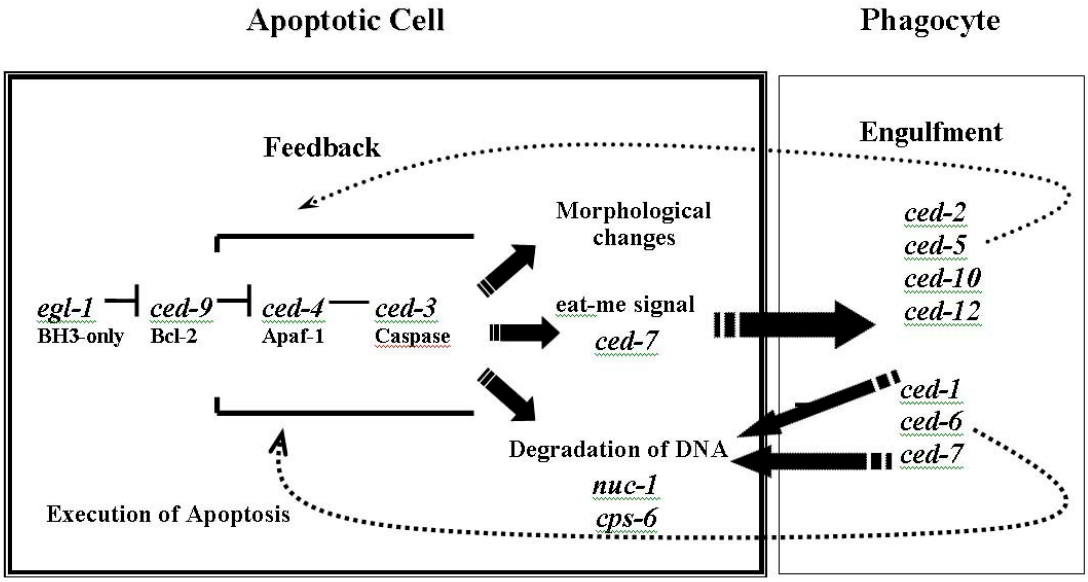


Figure 7 The genetic pathway of apoptosis in *C. elegans* [51]

6. Apoptosis in mammals

6.1 Caspases

The caspase, a cysteine protease homolog to *C. elegans* CED-3, are of central importance in the apoptotic signaling network which are activated in most cases of apoptotic cell death.[52] The term caspase is derived from cysteine-dependent asparate-specific protease, which has cysteine at the active site and specifically cleave after aspatic acid residue. Caspase synthesized as inactive proenzyme or zymogene. Conversion of each caspase precursor to the mature active form requires a proteolytic removal of prodomain

The apoptotic caspase can be divided into the group of initiator caspases (procaspase -2, -8, -9, -10) and executioner caspases or effector caspases (procaspase –3, –6, -7). Initiator caspases possess long prodomains that self activated by clustering or aggregation. While effector caspase possess only short prodomains. Effector caspases are

activated by initiator caspase. The caspase activation leads to degradation of specific target proteins. Most of the morphological changes of apoptosis are caused by caspases. Caspase activation can result from intrinsic pathway and extrinsic pathway.

6.2 Activation of caspases (stimulation of apoptosis)

Extrinsic apoptosis pathways (death receptor pathway)

Extrinsic signals stimulate the apoptosis via receptors. The receptors that trigger apoptosis are called death receptors. Death receptors are members of the Tumor necrosis factor receptor (TNFR) family. They all share similar cysteine rich extracellular domains which allow them to recognized their ligands with specificity, including TNFR-I, Fas / CD95 and the TRAIL receptor DR-4 and DR-5.[53] Ligands are bind to cell surface receptor cause trimerization of cell surface receptors and activation of the respective death receptor.[54] Subsequent signaling is mediated by the cytoplasmic path of the death receptor which contains a conserved sequence termed the death domain (DD). Adapter molecules like Fas-Associated Death Domain (FADD) or TRADD themselves, process their own DDs by which they are recruited to the DDs of the activated death receptor, thereby forming the so-called death inducing signaling complex (DISC). In addition to its DD, the adapter FADD also contains a death effector domain (DED) which through homotypic DED-DED interaction sequesters procaspase-8 to the DISC. Procaspase-8 is activated by autoproteolytic cleavage and forms the active caspase-8. Caspase-8 initiates a cascade of caspase activation leading to apoptosis by activate downstream effector caspases such as caspase-3, -6, -7.[55] (Fig. 8)

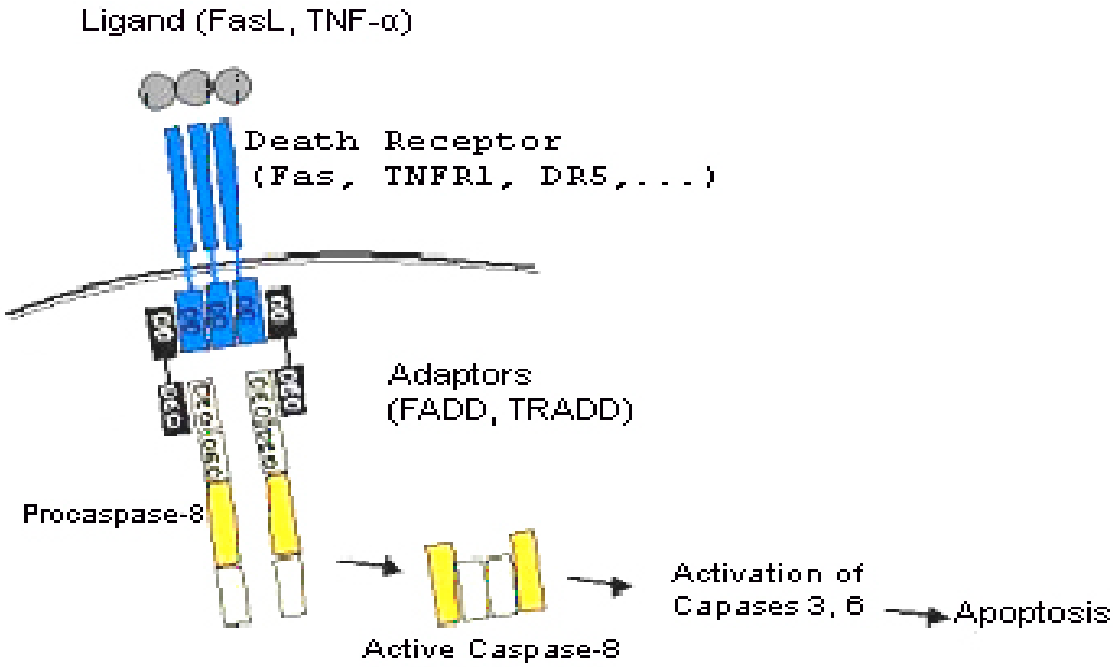


Figure 8 Extrinsic apoptosis pathways (death receptor pathway) [56]

Intrinsic apoptosis pathway (mitochondrial pathway)

Mitochondria plays a central role in the integration and propagation of death signal originated from inside the cell such as DNA damage, oxidative stress, starvation, as well as those induced by chemotherapeutic drugs.[57] Most apoptotic stimuli induce the release of the apoptogenic factors from mitochondria such as cytochrome C, which activates the apoptosome and therefore the caspase cascade, the apoptosis-inducing factor (AIF) and Smac/Diablo.[58-59] Permeability of the mitochondrial outer membrane is essential to the initiation of apoptosis through this pathway. Proteins belonging to the Bcl-*2* family appear to regulate the membrane permeability to ions and possibly to cytochrome C as well.[60] Although these proteins can themselves form channels in membrane, the actual molecular mechanism by which they regulate mitochondrial permeability and the solutes that are released are still unclear. The Bcl-*2* family is composed of a large group of anti-apoptotic members that when overexpressed prevent apoptosis and a large group of pro-apoptotic members that when overexpressed induce apoptosis. The balance between the anti-apoptotic and pro-apoptotic Bcl-2 family members may be critical to determining whether a cell undergoes apoptosis.

Suppression of the anti-apoptotic member or activation of the pro-apoptotic members of the Bcl-2 family leads to altered mitochondrial membrane permeability resulting in release of cytochrome C into the cytosol. Cytochrome C is bound by protein Apaf-1 (apoptotic protease activating factor-1), procaspase-9 and ATP to form the protein complex called the apoptosome. In this case, the dimerization of procaspase-9 molecules at the *Apaf*-1 scaffold is respondsible for caspase-9 activation. Once the initiator caspases have been activated they can proteolytically active the effector procaspase-3, -6 and –7. The active effector caspases then subsequently cleave a specific set of protein substrates, including procaspase themselves, resulting in the mediation and amplification of the death signal and eventually in the execution of cell death with all the morphological and biochemical features usually observed.[61](Fig. 9)

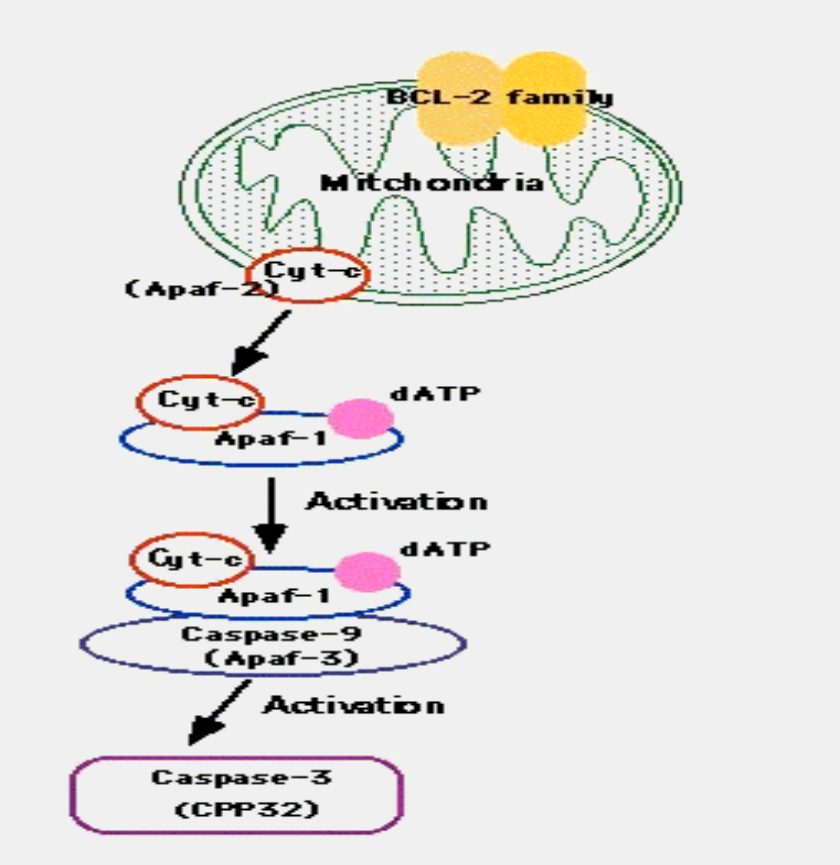


Figure 9 Intrinsic apoptosis pathways [62]

6.3 Control of caspase activation

The Bcl-2 family

Bcl-2, an oncogene in follicular lymphoma, is frequently linked to an immunoglobulin locus by the chromosome translocation (14:18) was the first example of an oncogene that inhibits cell death rather than promoting proliferation. The protein Bcl-2 appear localized or associated with intracellular membranes of mitochondrial, endoplasmic reticulum and nuclei.[63] They are assumed to function as ion channels, maintaining the integrity of the mitochondrial and preventing shedding of cytochrome C.

Bcl-2 family can be defined by the presence of conserved sequence motifs known as Bcl-2 homology domains (BH) which can be BH1-BH4. These domains are essential for the dimerizations of each member. All anti-apoptotic members e.g. Bcl-XL, Bcl-W contain all four domains BH1, BH2, BH3 and BH4. While the proapoptotic members are lack of BH4 domain. The proapoptotic members are divided into two subgroups: the Bax-subfamily consists of Bax, Bak and Bok that all possess the domains BH1, BH2 and BH3, whereas the BH3- only protein consists of Bid, Bim, Bik, Bad have only the short BH3 motif.[64-65]

The ratio of Bcl-2 and Bax in a cell determines apoptosis. When the Bcl-2 level is higher than the Bax level, apoptosis is suppressed. Alternatively, apoptosis is promoted when the Bcl-2 level is lower than the Bax level (Fig.10).

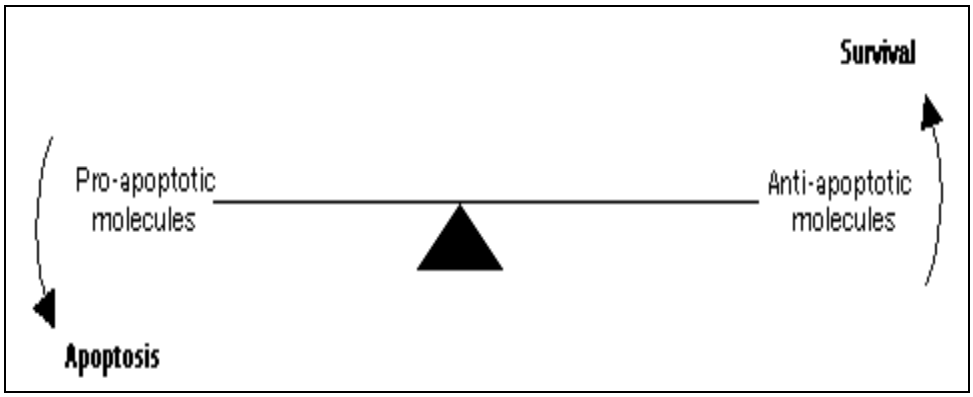


Figure 10 The balance of cell survival/death [66]

Suppressor of apoptosis by IAPs (inhibitors of apoptosis proteins)

The induction of apoptosis or progression through the process of apoptosis is inhibited by a group of protein called IAPs. IAPs are a family of antiapoptotic protein whose prototype originally was described in baculovirus with many homologues found to be conserved across several species. There have been 6 mammalian BIR containing proteins (BIRPs) identified.[67] These are inhibitor of apoptosis 1 and 2 (IAP-1) and (IAP-2), X-linked

inhibitor of apoptosis (XIAP), neuronal inhibitor of apoptosis (NIAP), BIR-repeat-containing ubiquitin-conjugating enzyme (BRUCE) and survivin. The BIR domain is necessary and sufficient for IAP function because it interacts with caspase that is believed to confer most the antiapoptotic activity of IAPs. Many member of IAP block proteolytic activation of caspase-3 and –7, thereby preventing initiation of the caspase cascade.[68]

Second Mitochondrial Activator of Caspases/Direct IAP Binding protein with Low pI (Smac / Diablo) the action of IAPs can be neutralized by the interaction of Smac/Diablo which is released form the mitochondrial intermembrane space.

7. Program cell death in *Drosophila*

Genetic studies on Drosophila have led to the identification of a gene called *reaper*, which plays a central role in initiation of cell death. Reaper transcripts are found in doomed cells, and their presence precedes morphological manifestations of apoptosis by 1 to 2 hr. The *reaper* gene apparently encodes a small peptide with no homologies to known proteins. Deletions of *reaper* suppress apoptosis in response to apoptotic stimuli. Thus, *reaper* mutant embryos contain many extra cells. *Reaper* embryos have an intact apoptotic pathway that fails to be initiated. Reaper is thought to integrate the information from diverse pathways and put a cell on the apoptotic pathway. At this point, the presence of *reaper* homologues in other organisms is unknown.[69]

8. Apoptosis in some parasites

Apoptosis is an essential part of cell biology and is thought to have evolved not only to regulate growth and development in multi-cellular organisms but also to have a functional role in the biology unicellular organisms. In protozoan parasite and other unicellular organisms, the feature of apoptosis is similar to those in multi-cellular organisms which have been reported. [70]

8.1 Apoptosis in *Leishmania*

Leishmania sp., a unicellular protozoan parasite caused a wide range of human disease from localized self – healing cutaneous lesions to fatal visceral infections which known as Lishmaniasis.

The studied of PhiPhiLux (PPL) gene of *L. donovani* by revealed that a certain fraction of PPL-cleavage activity is caspase–like enzyme. And the apoptosis process in *L. donovani* promastigotes follows a similar pathway as in higher eukaryotes, but there are some differences.

The induction of PPL-cleavage activity was observed in stationary phase promastigotes and axenic amastigotes and it is partially inhibited by caspase – specific inhibitors but not by the inhibitors of other cysteine proteases such as cathepsin or calpain.[71] The PPL is a cell permeable caspase-specific substrate (i.e. a peptide containing amino acids, DEVD) that fluoresces upon cleavage so that protease activity can be monitored in intact cells. PPL has been used to measure the level of caspase activity in higher eukaryotic cells.[72]

The induction of apoptosis in multicellular organisms follows a sequence of events, which include change in the mitochondrial potential resulting in release of factors (e.g. cytochrome c) into the cytosol.[73] The factors then activate the effector molecules such as caspases, which trigger downstream cleavage of cellular targets and DNA cleavage followed by change in the plasma membrane integrity.[74] Similarly mitochondrial membrane potential, PPL-cleavage activity and plasma membrane permeability in *L. donovani* promastigotes was measured during active and the stationary phase of growth.[71] There was first a drop in the mitochondrial membrane potential, followed by simultaneous induction of the caspase–like activity and change in the plasma membrane integrity in stationary phase promastigotes.[71] Taken together, these results suggest that apoptosis in *L. donovani* promastigotes follow a similar pathway as in higher eukaryotes, however with some differences.

8.2 Apoptosis in *Trypanosomes*

In *T. brucei rhodesiense*, apoptosis was shown to correlate with the induction of two genes, prohibitin and a receptor for activated protein kinase C.[75] Prohibitin, a proto-oncogene, is involved in cell cycle control and senescence of higher eukarytic cells.[76] Similarly, receptors for activated protein kinase C have been shown to play an important role in apoptosis in higher eukaryotes as well.[77] The correlation between changes in the expression of the two genes and the apoptosis pathway in *T. brucei rhodesiense* suggests some similarity in the induction of apoptosis in trypanosomes and in higher eukaryotes.

In *T. brucei*, activation of caspase-independent apoptosis by reactive oxygen species was shown to be due to alteration in Ca^{2+} levels in mitochondria.[78] The expression of the mammalian anti-apoptotic protein *bcl-2* in this parasite did not reverse the mitochondrial dysfunction as it does in mammalian cells.[78] Thereby suggesting that proteins involved in Trypanosome apoptosis may be distinct from those in metazoans. Recently, five orthologs of caspases known as metacaspased have been idenified in the *T. brucel* genome (www.tigr.org). Expression of *T. brucei* metacaspase 4 in yeast resulted in growth inhibition, mitochondrial dysfunction and clonal death of the yeast.[74] Further, mutations in the putative catalytic dyad residues histidine and cysteine of *T. brucei* metacapase 4 reversed the effects thereby confirming that it is a cysteine proteinase.[79]

8.3 Apoptosis in *Plasmodium*

Apicomplexan protozoa of the genus Plasmodium are the causative agents of malaria. During its life cycle, the malaria parasite goes through several stages of differentiation in harsh environments resulting in cell death of many parasites.[80] Cell death in the erythrocytic stages of chloroquine treated *Plasmodium falciparum* was shown to be due to apoptosis as indicated by oligonucleosomal DNA fragmentation.[81]

Cell death of *P. berghei* undergoing defferentiation from zygotes to ookinetes exhibit typical features of metazoan apoptosis including condensation of chromatin, fragmentation of the nuclear DNA and change in plasma membrane permeability. In addition, caspase-like activity was also identified in the cytoplasm of ookinetes, which was blocked by caspase inhibitors. Partial characterization of the caspase-like activity revealed that it exists in two parasite proteins of approximately 45 and 28 kDa. The treatment of both zygotes and ookinetes of *P. berghei* with caspase inhibitors *in vitro* prevented cell death and resulted in doubling of the number of parasites.[82]

CHAPTER 3

MATERIALS AND METHODS

1. Parasite collection

The *B.malayi* infected blood samples were collected from infected-cat from Narathiwat in 2003. The infected blood samples were stored at 4 ^{o}C until used.

2. Isolation of parasites [83]

Five milliliters of microfilaria-infected blood was intravenously taken from each patient and animal and transferred to a 10 ml-test tube containing 7 mg/ml of EDTA as an anti-coagulant agent. The blood was diluted with equal volume of phosphate buffered saline, pH 7 (PBS; 0.137 M NaCl, 10 mM Na_2HPO_4, 3.2 mM KH_2PO_4) and filtered through a 5 μm polycarbonate membrane (Millipore). Freely moving microfilariae were then soaked in PBS and washed three times with the same buffer prior to centrifugation at 5,000 rpm for 10 min. The pellets of microfilariae were stored at -70^{o}C until used.

3. Identification microfilaria

GIEMSA staining was used for microfilarial identification. Briefly, microfilaria was separated by hemofiltration using polycarbonate membrane (Millipore) with pore size of 5 mm to eliminate RBC and most WBC. The filtrated microfilaria was smeared on a clean slide. A film was allowed air dry thoroughly for several hours. Air-dried film was fixed by soaking the film for 3 min in a Coplin jar containing absolute methanol for 2 – 3 min and then stained with diluted Giemsa stain (1:50 v/v) for 45 min. The stained slide was washed in buffered water and let air dry in a vertical position. The slide was determined under light microscopic examination

4. Isolation of genomic DNA

The genomic DNA was extracted from filarial parasites using genomic DNA Purification Kit (Gentra). The procedure of isolation was performed according to the instruction manual provided by the company. The microfilaria pellet was digested in 300 μl

of Cell Lysis Solution (0.01 M Tris-2%, SDS-0.01 M, EDTA pH 8) and 1.5 μl of Proteinase K Solution (200 mg/ml) at 55 ^{o}C for 4-6 hrs. The lysate was further digested by the addition of 0.12 units of RNase A Solution (1 mg/ml in 5 mM Tris-HCl, pH 8.0) and incubated at 37 °C for 15-60 min. A 100 μl of Protein Precipitation Solution (3 M ammonium acetate pH 7.4) was added to the cell lysate, quickly spinned at high speed for 20 sec and centrifuged at 13,000 rpm for 3 min. Supernatant containing DNA was transferred to a new tube, precipitated by the addition of 300 μl of 100% isopropanol, and centrifuged at 13,000 rpm for 3 min. The supernatant was removed and DNA pellet was washed once with 300 μl of 70% ethanol. The sample was mixed and centrifuged at 13,000 rpm for 3 min. DNA pellet was dried (air-dry) for 10 min, dissolved in 20 μl of DNA Hydration Solution (TE; 0.01 M Tris-0.001 M, EDTA pH 7.4) and incubated at 65 °C for 60 min. The genomic DNA of parasites was stored at 4 °C until use. DNA concentration was determined using UV spectrophotometer and the absorbance was measured at 260 nm. Molar extinction of DNA was calculated as; 1 O.D. 260 nm = DNA concentration of 50 ng/ml.

5. Detection microfilaria DNA

The detection of *B. malayi* were designed based on the conserved region of brugia sp. SLX-F/SLX-R primers were used for detection *B. malayi* DNA by the trans-spliced leader exon a target sequence.

6. PCR amplification

6.1 Primer

The sets of primer were used for amplification of the gene encoding for apoptosis from *B. malayi.* The primers were designed based on the nucleotide sequence of apoptosis genes of *C. elegan*s. (Table 1)

Table 1 List of primers used for PCR amplification

ced-3 gene

Sequence Name	Nucleotide sequence	Tm(°C)	Product (bp)
C3PF	TTY GTN CAR GCN TGY MGN GG	56	298
C3PR	GCY TGN AGR AAC CAN GAN CC	57	
C3N1F	GAA CTG GAA GGA AGA GGA AA	58	569
C3N1R	GCA CAG TTC CGC AGA TTC	54	
C3N2F	TCT GAA ATA AGG TGA TAA T	50	540
C3N2R	AGG TCG GAA ATC TTA GGA	52	
C3N3F	GTA TCC TCA ACT TGT CCC G	58	627
C3N3R	TAC TGT AGC GTT GTG ACG AT	58	
C3N4F	ATC TGA AAT ATA GCC TGA	49	638
C3N4R	TCC TCA ACA AAT CTA TCT C	57	
C3N5F	AGG ACA TAA AAA TCA GTA	46	384
C3N5R	CCT AAA TAT TAT TAT CAA	46	

ced-4 gene

Sequence Name	Nucleotide sequence	Tm(°C)	Product (bp)
C4N1F	ATG CTC TGC GAA ATC GAA TG	58	750
C4N1R	CAA CTG ACG TGA CAT GCT C	58	
C4N2F	TGA CTG TAT CTA AAG GCA CC	57	530
C4N2R	TCA CAC AGG AAA CAG CTA T	55	
C4C1F	CGG TCT CGG GTA TGT CTT TA	62	371
C4C1R	ATT CGT CGT TTC GTA AGG TT	61	
C4C2F	AAT GGA ACC AAG GGA CAA	52	492
C4C1R	CAC TCT ATG GAC CGC ACC	53	

ced- 9 gene

Sequence Name	Nucleotide sequence	Tm(°C)	Product (bp)
C9N1F	GCA AAG ACG CCA ATC CA	56	489
C9N1R	CAA GAA TAG CCG AAA GTC C	56	
C9N2F	GCA AAG ACG CCA ATC CA	56	340
C9N2R	CTT GAA GAC AGC GGT AAC A	56	

6.2 Amplification of apoptotic gene by standard PCR condition

A standard procedure for PCR amplification was performed in 20 μl volume. The reaction contains 50 ng of genomic DNA in 1x PCR buffer, 2.0 – 3.0 mM $MgCl_2$, 0.3 mM dNTP, 0.5 – 2.0 μM each of primers, 1 unit of *Taq* DNA polymerase and distilled water. The PCR amplification was performed using DNA thermal cycle apparatus. Each cycle consist of pre-denaturation at 94 ^{o}C for 4 min, denaturation at 94 ^{o}C for 1 min, annealing (temperature vary depend on each primer) for 45 sec , extension at 72 ^{o}C for 1 min and Post-extension at 72 ^{o}C for 4 min. The PCR products were analyzed using electrophoresis in 1.5-1.6% agarose gel at 110 volt for approximately 30 min prior to ethidium bromide staining and record the DNA band under ultraviolet light.

7. Cloning of the PCR fragments

7.1 Purification of PCR products

The PCR products were purified by (ROCHE®) high pure PCR product purification kit. The procedure of purification was performed according to the instruction manual provided by company. After PCR amplification is complete, adjust total volume for each PCR tube to 100 μl and 500 μl of binding buffer PCR sample was mixed and added with placed into a high pure filter tube, centrifuged at 12,000 rpm for 60 seconds, discarded flow-through solution and placed filter tube back in the same collection tube followed by adding 500 μl of washing buffer to the upper reservoir and centrifuge at 12,000 rpm for 60 seconds, discard the flow-through solution. After that, 200 μl of washing buffer was added and centrifuged at 12,000 rpm for 60 seconds, discard the flow-through solution 50-100 μl of elution buffer then added to the upper reservoir of the filter tube which on placed to a

clean 1.5 ml microcentrifuge tube and centrifuged at 12,000 rpm for 60 seconds. The upper reservoir was removed and the purified DNA was collected from the microcentrifuge tube.

7.2 Purification of PCR fragments

The PCR fragment was eluted from the gel and purified by using QIAGEN® Purification system prior to cloning. The procedure of purification was performed according to the instruction manual provided by the company. The DNA fragment was excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in a colorless tube and added 3 volumes of Buffer QG (guanidine thiocyanate) to 1 volume of gel. Then, the gel slice was incubated at 50 °C for 10 min until the gel slice was completely dissolved. After that, the color of the mixture which should be yellow was checked. The mixture was placed into a QIAquik spin column, centrifuged at 10,000 rpm for 1 min, discarded flow-through and placed QIAquick column back in the same collection tube. After that, the DNA bound QIAquick column was washed with 0.5 ml of Buffer QG and centrifuged at 10,000 rpm for 1 min, followed by washing with 0.75 ml of Buffer PE (Sodium acetate, 80% ethanol) to QIAquick column and centrifuged at 10,000 rpm for 1 min. The DNA bound QIAquick column was centrifuged again at 10,000 rpm for 1 min and placed QIAquick column into a clean 1.5 ml microcentrifuge tube. The DNA was eluted with 50 μl of Buffer EB (10 mM Tris-Cl, pH 8.5). The column was centrifuged at 10,000 rpm for 2 min.

7.3 Ligation of PCR fragment and vector

The purified 200 ng of PCR fragment was further ligated to the pGEM®-T Easy vector using protocol of pGEM® -T Easy Vector Systems (Invitrogen, Promega). The reaction was consisted of 4 μl of PCR product, 1 μl of 2X Rapid Ligation Buffer; T4 DNA Ligase (60mM Tris-HCl, pH 7.8, 20mM $MgCl_2$, 20mM DTT, 2mM ATP 10% PEG), 1 μl of the pGEM®-T Easy vector and 1 μl of T4 DNA Ligase (3 Unit of final concentration). The reaction was mixed, and incubated for 12-14 hrs at 4 °C.

7.4 Preparation of ultra-competent *E. coli* cells for transformation

The competent *E. coli* cells were prepared for transformation. Single colony of selected *E. coli* strain (DH5α) was cultured in 5 ml of LB-broth and incubated for 12-14 hrs at 37 °C with moderate agitation (~250 rpm). Then, 1 ml of the overnight culture was added to 150 ml of LB-broth and grown at 37°C with moderate agitation until lag phase. (the OD_{600} nm ≈ 0.5) Then, the culture was placed on ice for 10 min and centrifuged at 5,000 rpm for 10 min at 4°C. The bacteria pellet was washed twice in 10 ml and 5 ml of

sterile 10% by centrifugation at 5,000 rpm for 10 min at 4°C. After that, the pellet was resuspended in 0.02-0.2 ml of cold 10% glycerol, and aliquots of 40 µl was transferred to a new eppendorf tubes for further transformation or kept at -70°C until used.

7.5 Transformation by electroporation

Electroporation transformation was performed by using BioRad MicroPulser (BIO-RAD MicroPulser™) according to the protocal provided by the company. BioRad MicroPulser was set to "Ec 2" for 2 mm cuvettes (2.50kV). Four microliters of the ligation product was added to the competent cells. Transformation reaction was transferred to a chilled electroporation cuvette, and immediately pulsed in the electroporator. Then, 250 µl of LB broth was added to the reaction. The mixture was transferred into 1.5 ml eppendorf tubes and incubated at 37 °C for 45 min. After that, 100 to 150 ul of the mixture was spread on the LB-ampicillin agar plates containing X-gal and IPTG (40 µl of 20 mg/ml X-gal and 10 µl of 200 mg/ml IPTG were spread on the LB-ampicillin agar plates 30 minutes prior to plating the transformations). The plates were incubated for 17 hrs at 37 °C. Colonies containing plasmids without inserts were blue color. Colonies containing plasmids with inserts remained white and were selected for examination by transferring to a new LB–ampicillin agar plate containing X-gal and IPTG for verification of the phenotype.

8. Recombinant DNA extraction

White colonies of the recombinant DNAs were selected and cultured in 5 ml of LB media in the presence of 100 µg/ml of ampicilin for 24 hrs. The recombinant DNAs were purified using QIAGEN® QIAprep Spin Miniprep Kit Protocol. The procedure of extraction was performed according to the instruction manual provided by the company. The *E. coli* cells containing recombinant DNA were grown in 5 ml of LB broth for 12-14 hrs at 37 °C. Each culture was transferred to a microcentrifuge tube and centrifuged for 1 min at 5,000 rpm. The supernatant was removed and cell pellet was resuspended in 250 µl of Buffer P1 (2 mg/ml lysozyme, 50 mM glucose, 10 mM CDTA (cyclohexane diamine tetracetate), 25 mM Tris-HCl, pH 8.0). After resuspension of the pellet, 250 µl of Buffer P2 (sodium hydroxide) was added to the mixture and the tube was gently inverted for 4-6 times. Then, 350 µl of Buffer N3 (guanidine hydrochloride, acetic acid) was added and the solution was mixed gently but thoroughly, immediately after addition of Buffer N3. All reactions were centrifuged at 13,000 rpm for 10 min. Then, the supernatant was applied from top step into

the QIAprep Spin Column, centrifuged at 10,000 rpm for 1 min. After that, QIAprep Spin Column was washed by the addition of 0.5 ml of Buffer PB (guanidine hydrochloride, isopropanol) followed by centrifugation at 10,000 rpm for 1 min. Subsequently, QIAprep Spin Column was washed with 0.75 ml of Buffer PE Sodium acetate, 80% ethanol and centrifuged at 10,000 rpm for 1 min. The flow-through was discarded and the column was placed for centrifugation at 10,000 rpm for 1 min. The QIAprep column was transferred a clean 1.5 ml microcentrifuge tube and DNA was eluted with 20 μl of Buffer EB (10 mM Tris-Cl, pH 8.5) prior to centrifugation again at 10,000 rpm for 2 min.

9. Analysis of inserted apoptosis genes in recombinant DNA

The purified DNAs were tested by PCR amplification and restriction enzyme digested. The PCR reaction was performed according to the methods described above. Restriction enzyme digestion restriction was manipulated in 20 μl volume containing 100-200 ng of the purified recombinant DNA, 1x buffer and 10 unit of *Eco*R I (GIBCOBRL®). The reaction was subsequently incubated at 37 °C for 2 hrs followed by heating at 68 °C for 10 min. The PCR product or the digested product was analyzed by electrophoresis on 1.2% agarose gel at 110 Volts prior to ethidium bromide staining and observation under ultraviolet light.

10. DNA sequencing of apoptotic genes

The DNA sequence analysis was performed by BSU (Bioservice Unit), National Science and Technology Development Agency (NSTDA) as followed;

DNA sequencing was using Big Dye Terminator Cycle Sequencing procedure and analyzed using ABI PRISM 3100 (Perkin Elmer). The PCR condition for DNA sequencing was followed by 25 cycles of denaturation at 95 °C for 5 secs, annealing at 50 °C for 4 secs and extension at 60 °C for 4 min. The nucleotide sequencing data was analyzed by using software of ABI PRISM Model 3100 version 3.7, analysis procedure was accomplished according to the manual instruction provided from company.

11. Complete sequence of apoptosis genes

The nucleotide sequence data was analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The percentage of nucleotide identity in comparison

to various organisms was reported. The sequence data of apoptosis genes of *B. malayi* were analyzed for their identity to *B. malayi* genome (http://www.tigr.org). All sequence data were analyzed using Clustal W sequence alignment program of the European Molecular Biology Laboratory (EMBL,U.K.) (http://www.ebi.ac.uk/). Thereafter, the sequence data with high identity was analyzed for open reading frame using the ORF finder program (http://www.ncbi.nih.gov/gorf/gorf).

CHAPTER 4

RESULTS

1. *B. malayi* genomic DNA extraction

The genomic DNA of *B. malayi* was extracted and analyzed by agarose gel electrophoresis. A single band of high molecular weight DNA sized more than 23 kb was observed after ethidium bromide staining. The DNA concentration was estimated by spectrophotometric measurement at wavelength of 260 nm. In the Figure 11 the DNA concentration was 60 ng/ml and was used for isolating putative apoptotic gene by PCR amplification.

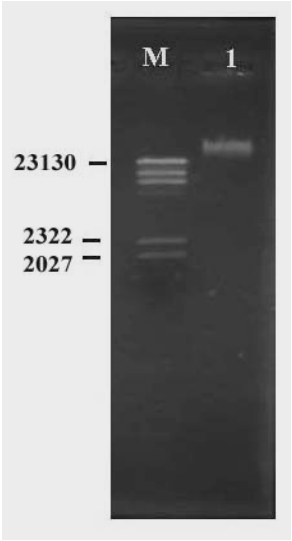


Figure 11 Ethidium bromide staining of 0.8% agarose gel electrophoresis of *B. malayi* genomic DNA.

Lane M: Lamda *Hind* III DNA marker

Lane 1: 60 ng of *B. malayi* genomic DNA

2. Identification microfilaria

2.1 Giemsa stain

The Brugia parasite was identified by staining with Giemsa. In Figure 11 confirmed that the parasite was *B. malayi*. Because *B. malayi* have terminal nuclei, nucleus column overlapping and have the cephalic space length to width ratio at 2:1. (Figure 12)

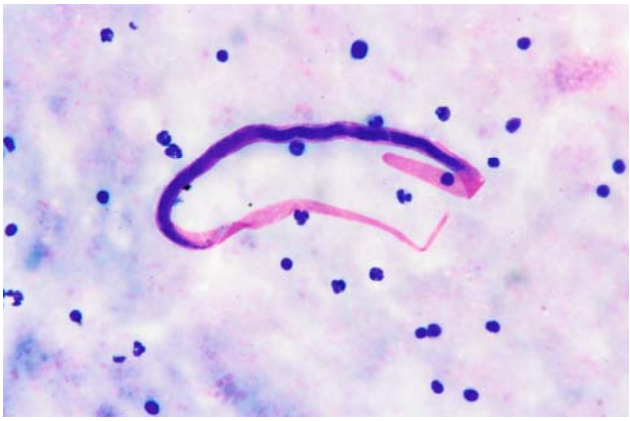


Figure 12 Giemsa staining of of *B. malayi* extracted from blood of infected cat.

2.2 Detection of microfilaria DNA by PCR

The PCR amplification of extracted DNA was performed by using SLX-F/SLX-R primer. The primers amplified 294 bp DNA fragment which specific for *B. malayi* confirmed that the DNA used in this study was from *B. malayi.* (Figure 13)

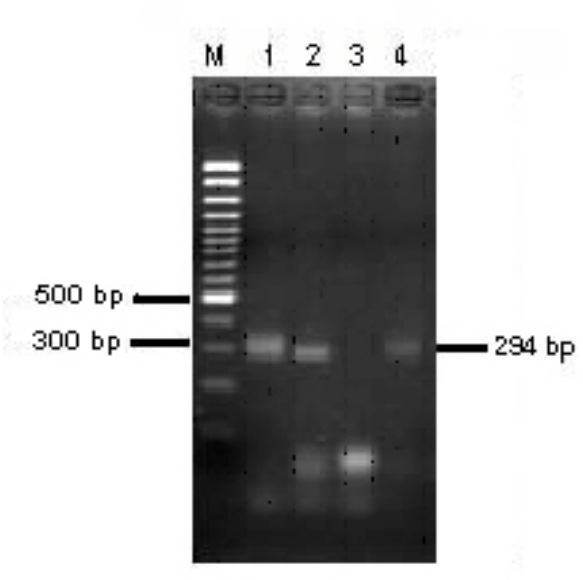


Figure 13 Electrophoretic separation of PCR product amplification from genomic DNAs using SLX-F/SLX-R primers.

Lane M = 100 bp Ladder plus

Lane 1 = *B. pahangi* DNA

Lane 2 = *B. malayi* DNA

Lane 3 = cat DNA

Lane 4 = *B. malayi* DNA from infected cat

3. PCR amplification of apoptotic genes of *B. malayi*

The *B. malayi* DNA was used as a template for PCR amplification using primers that specific to the genes which induce and inhibit apoptosis process in *C. elegans*, the close related organism. The PCR conditions were initially optimized for each primer set

3.1 Optimization of the PCR condition

The optimum condition was performed for efficient amplification of apoptotic genes of *B. malayi.* In this study, the primers were designed from the nucleotide sequence of apoptotic genes of *C. elegans* including *ced-3*, *ced-4* and *ced-9.* Several primer sets that specific for each apoptotic gene was examined for optimum condition such as annealing temperature, primer concentration, and magnesium ion concentration. The optimum conditions for each primer were reported in Table 2 to 4.

For *ced-3* gene, the optimum conditions of the three primer sets were shown in Table 2.

Table 2 The optimum condition of PCR primer sets for isolating putative *ced-3* gene of *B. malayi*

Primer Name	Primer concentration	Magnesium ion concentration	Annealing temperature
C3N2F/ C3N2R	1.0 μM	2.5 mM	46 °C
C3N3F/ C3N3R	2.0 μM	2.0 mM	47 °C
C3N4F/ C3N4R	2.0 μM	2.5 mM	45 °C

The other sets of primers including C3PF/ C3PR, C3N1F/ C3N1R and C3N5F/ C3N5R were performed in various conditions but no amplification product was obtained.

For *ced-4* gene, all of the designed primer sets could amplify the parasite DNA. The PCR condition for each primer set was summarized in Table 3.

Table 3 The optimum condition of PCR primers for isolating putative *ced-4* gene of *B. malayi*

Primer Name	Primer concentration	Magnesium ion concentration	Annealing temperature
C4N1F/ C4N1R	1.0 μM	2.0 mM	54 °C
C4N2F/ C4N2R	1.0 μM	1.5 mM	46 °C
C4C1F/ C4C1R	1.0 μM	2.5 mM	45 °C
C4C2F/ C4C2R	1.0 μM	2.0 mM	44 °C

The optimum condition for amplification of *ced-9* gene from *B. malayi* was shown in Table 4.

Table 4 The optimum condition of PCR primer set for isolating putative *ced-9* gene of *B. malayi*.

Primer Name	Primer concentration	Magnesium ion concentration	Annealing temperature
C9N1F/ C9N1R	1.0 μM	2.0 mM	48 °C
C9N1F/ C9N2R	1.5 μM	2.0 mM	48 °C

3.2 PCR amplification of putative *ced-3* gene from *B. malayi* DNA

The primers of *ced-3* gene were designed from 6 regions of *ced-3* gene of *C. elegans*. In Figure 13 A-C showed the analysis of PCR products resulted from PCR amplification. PCR product resulted from C3N2F/C3N2R amplification gave several DNA fragments sized between 300 bp to 1000 bp with the major band at 600 bp (Figure 14A). PCR products of C3N3F/C3N3R amplification were 300 bp, 350 bp, 500 bp, and 700 bp in length (Figure 14B). The amplification using C3N4F/ C3N4R primers gave a single DNA fragment at 650 bp (Figure 14C).

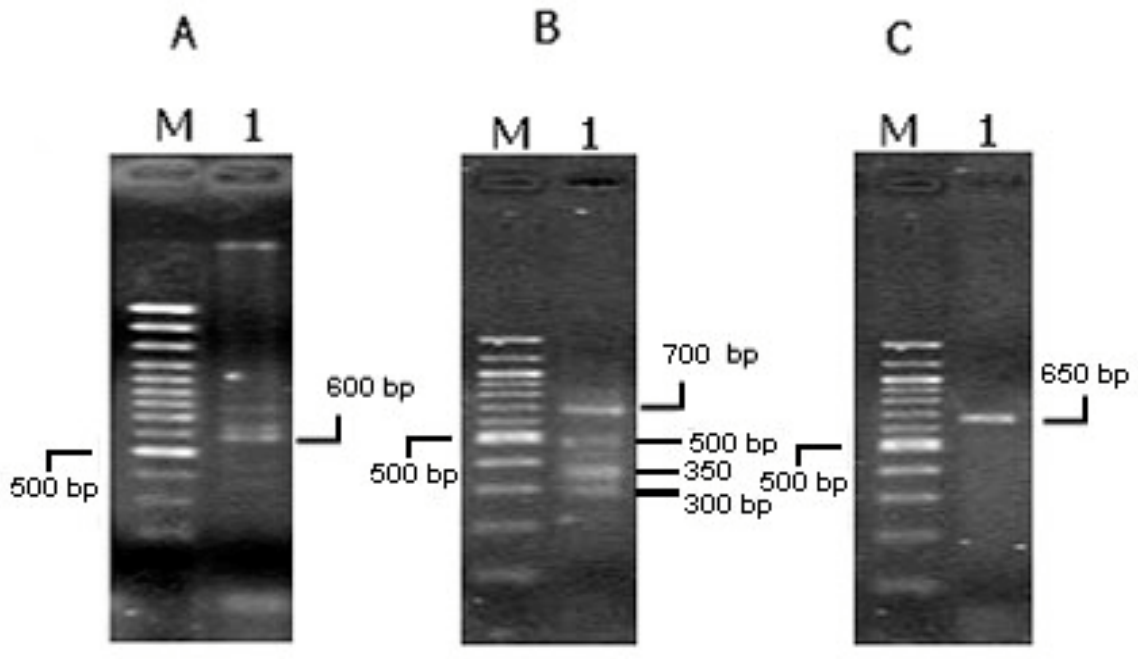


Figure 14 Ethidium bromide staining of 1.2%agarose gel electrophoresis of PCR product amplified by using C3N2F/R (A), C3N3F/R (B), and C3N4F/R (C) primers.

3.3 PCR amplification of putative *ced-4* gene from *B. malayi*

The primer sets, C4N1F/ C4N1R and C4N2F/ C4N2R, were designed from nucleotide sequence of *ced-4* gene of *C. elegans*. The analysis of PCR product resulted from C4N1F/ C4N1R amplification were DNA fragments sized between 300 to 900 bp with two dominant bands at 700 bp and 900 bp (Figure 15A). The PCR product of C4N2F/ C4N2R primers showed DNA fragments between 400 bp to 1,000 bp (Figure 15B).

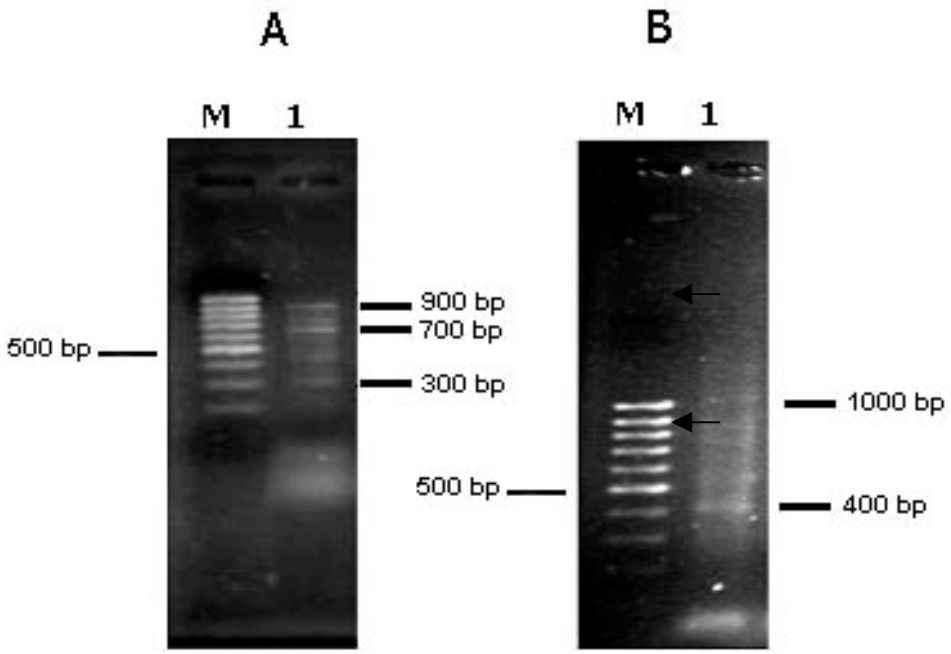


Figure 15 Ethidium bromide staining of 1.2%agarose gel electrophoresis of PCR products amplified by using C4N1F/R primers (A), and C4N2F/R primers (B).

3.4 Re-amplification of putative *ced-4* gene from *B. malayi*

Two primer sets, C4C1F/ C4C1R and C4C2F/ C4C2R which designed from nucleotide sequence of PCR product of C4N1F/ C4N1R at 474 and 591 were used to re-amplified the *B. malayi* DNA for more specific product. The amplification using C4C1F/ C4C1R primers gave two DNA fragments sized 500 bp and 1000 bp (Figure 16A), whereas the PCR product from C4C2F/ C4C2R showed major band at 350 bp (Figure 16B).

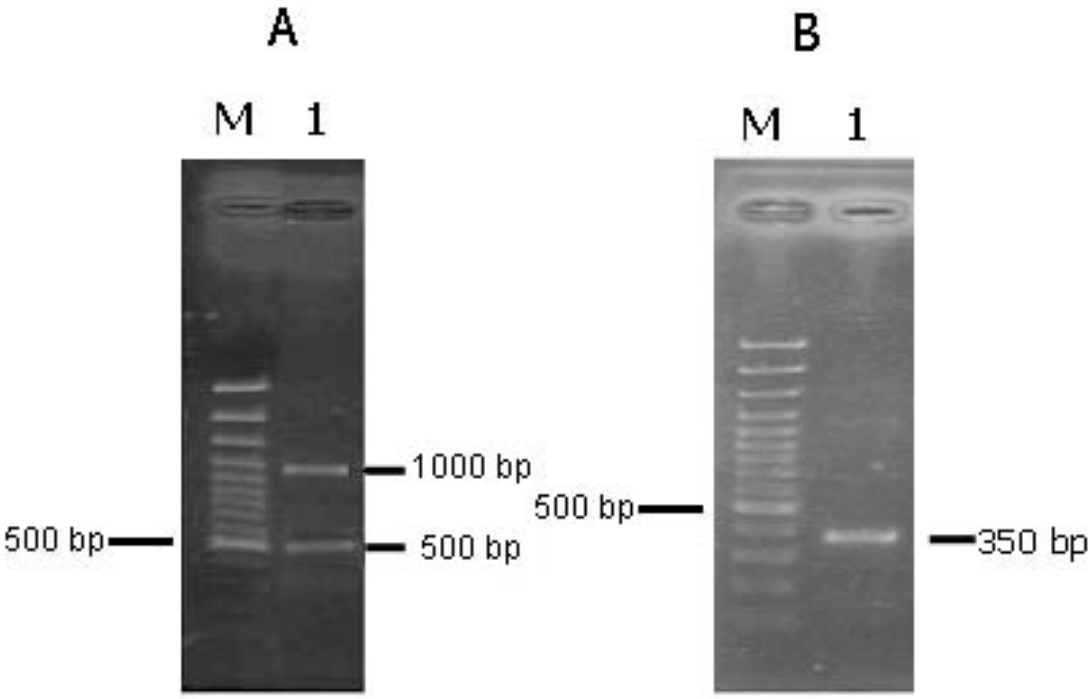


Figure 16 Ethidium bromide staining of 1.2%agarose gel electrophoresis of PCR products amplified by using C4C1F/ R (A), and C4N2F/ R primers (B).

3.5 PCR amplification of putative *ced-9* gene from *B. malayi*

The primers, C9N1F/ C9N1R and C9N1F/ C9N2R were used to amplify *ced-9* gene of *C. elegans*. The size of PCR products from C9N1F/ C9N1R were ranged from 300 bp to 1,000 bp. C9N2R was designed from DNA sequence between C9N1F/ C9N1R and C9N1R primer (Figure 17A). PCR Product sizes of C9N1F/C9N2R were observed at 450 bp and 600 bp (Figure 17B).

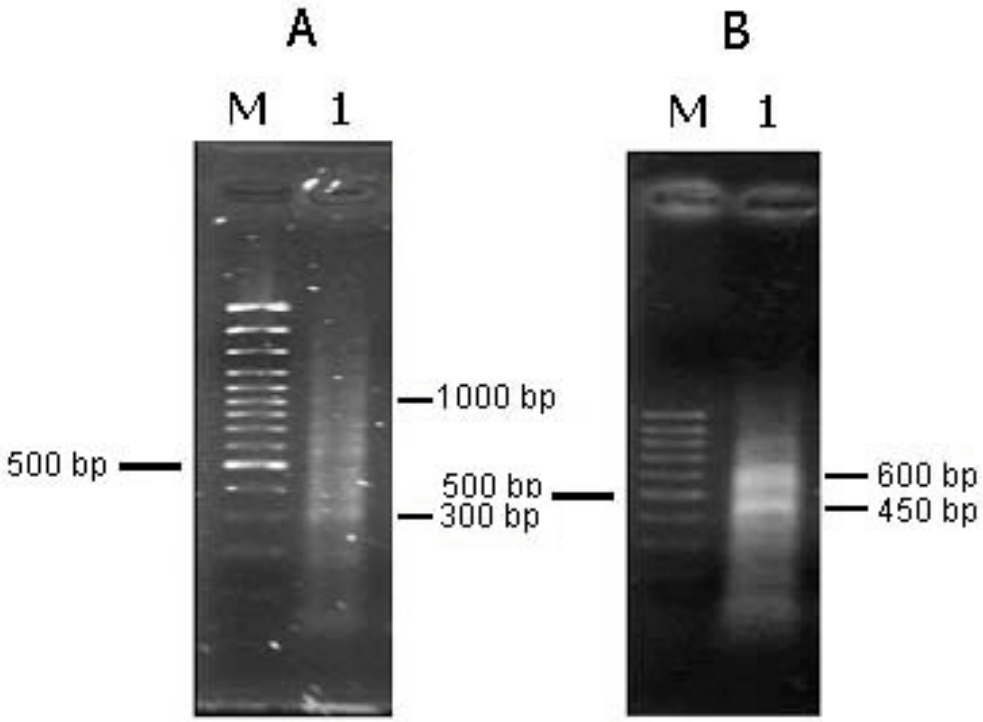


Figure 17 Ethidium bromide staining of 1.2%agarose gel electrophoresis of PCR product amplified by using C9N1F/ R (A) and C9N2F/ R primers (B).

4. Cloning and DNA sequencing of putative apoptotic genes from *B. malayi*

The PCR products from putative *ced-3, ced-4, and ced-9* genes of *B. malayi* were purified and ligated into the pGEM® - T Easy vector (Promega). The white colonies were picked and cultured in LB medium containing ampicilin. The selected recombinant plasmids were sequenced. The length of DNA fragments amplified from each primer are shown in Table 5.

Table 5 The list of primers and sized of DNA fragments from PCR amplification.

Apoptotic gene	Primer	Size of insert (bp)
Ced-3 gene	C3N2F/ C3N2R	686
	C3N3F/ C3N3R	455
	C3N4F/ C3N4R	569
Ced-4 gene	C4N1F/ C4N1R	474, 733 , 480 , 591
	C4N2F/ C4N2R	343
	C4C1F/ C4C1R	363, 416
	C4C2F/ C4C2R	270
Ced-9 gene	C9N1F/ C9N1R	712
	C9N1F/ C9N2R	468, 524

5. Analysis of the nucleotide sequences

The nucleotide sequence data of putative *ced-3, ced-4, and ced-9* genes of *B. malayi* were analyzed for their similarity and identity with apoptosis genes of *C. elegans* and the gene in *B. malayi* genome by using Clustal W program. The results were summarized in Table 6-8. The multiple alignments of each nucleotide sequence with highest identity are shown in Figure 18-23.

Table 6 Comparison of nucleotide sequences of putative *ced-3* to *ced-3* of *C. elegans* gene and *B. malayi* genome.

Primer	No. nucleotide	*C.elegans*		*B.malayi*	
	bp	Gene	% Identity	Gene code	% Identity
C3N2F/R	686	*ced-3*	48	BRNLH52TF	48
C3N3F/R	455	*ced-3*	41	-	-
C3N4F/R	569	*ced-3*	52	BRUKX73TF	96

Table 7 Comparison of nucleotide sequence of *ced-4* with *C. elegans ced-4* gene and *B. malayi* genome.

Primer	No. nucleotide	*C.elegans*		*B.malayi*	
	bp	Gene	% Identity	Gene code	% Identity
C4N1F/ R	480	*ced-4*	38	-	-
	474	*ced-4*	53	BRHDH13TF	92
	591	*ced-4*	49	BRPIR88TF	43
	733	*ced-4*	48	BRBAO18TJ	45
C4N2F/ R	343	*ced-4*	44	-	-
C4C1F/ R	363	*ced-4*	44	-	-
	416	*ced-4*	47	-	-
C4C2F/ R	270	*ced-4*	42	-	-

Table 8 Comparison of nucleotide sequence of putative *ced-9* with *C. elegans ced- 9* gene and *B. malayi* genome.

Primer	No. nucleotide	*C.elegans*		*B.malayi*	
	bp	Gene	% Identity	Gene code	% Identity
C9N1F/ R	712	*ced-9*	46	BRIK560TR	98
C9N1F/2 R	468	*ced-9*	41	BRPV162TR	48
C9N1F/2 R	524	*ced-9*	45	BRUGC95TR	48

```
CLUSTAL W multiple sequence alignment

BRUKX73TF       TATTATTGAAGTTGTTAAATGACAACACCGT-ATATGACCATTTCTCTTGACTGCTAAAT 179
C3N4F/R 569 bp  ----ATTGAAGTTGTTAAATGACAACACCGGGATATGGCCATTTCCCTTGGTTGCTAAAT 56
                    *************************  ***** ******* ****  ********

BRUKX73TF       AATCAATCAATTTAAAAATATCTTTTTTTTTTTTAAATGAATTGCTATTTTTCAATTGTT 239
C3N4F/R 569 bp  AATCAATTAATTTAAAAATATCTTTTTTTTAATTAAATGAATTGCTATTTTTCAATTGTG 116
                ******* **********************  **************************

BRUKX73TF       TTAACAATTAATTAAAACAAAATTTGATAATACTAGAATTTATTTCCTAGTTTAATATAC 299
C3N4F/R 569 bp  TTAACAATTAATTAAAACAAAATTTGATAATACTAGAATTTATTTCCTAGTTTAATATAC 176
                ************************************************************

BRUKX73TF       AAAAGCTCTTAAAAATTGTAGGAATTTCATAAAATCAATACTTGAATTTGAAATAAAAAT 359
C3N4F/R 569 bp  AAAAGCTCTTAAAAATTATAGGAATTTCATAAAATCAATACTTGAATTTGAAATAAAAAT 236
                ***************** ******************************************

BRUKX73TF       TAATTTTTATTTTAAAAATGATTTAAAACTATGAAAAATTAGAAATTTTATTAATTTCGC 419
C3N4F/R 569 bp  TAATTTTTATTTTAAAAATGATTTAAAACTATGAAAAATTAGAAATTTTATTAATTTCGC 296
                ************************************************************

BRUKX73TF       TACTGAATTACTATTCAAAAGTTAATTACTAAAGTTTTTTTTATTATTTAAGATGTGAAA 479
C3N4F/R 569 bp  TACTGAATTACTATTCAAAAGTTAATTACTAAAGTTTTTTTTATTATTTAAGATGTGAAA 356
                ************************************************************

BRUKX73TF       TGACCACAACACATATGATAAATTCCTGCGTAACGCATATAAAATAATCAATTGAGTCCT 539
C3N4F/R 569 bp  TGACCACAACACATAGGATAAATTCCTGCGTAACGCATATAAAATAATCAATTGAGTCCT 416
                *************** ******************************************

BRUKX73TF       AAATATTTATTTTTTTTAATGATTCATTTCTATAATTGAAATTTATTTTTATGTCAAATT 599
C3N4F/R 569 bp  AAATATTTATTTTTTTTAATGATTCATTTCTATATTTGAAATTTATTTTTATGTCAAATT 476
                ********************************** ************************

BRUKX73TF       TAGATTTTGATATTAATGAAAATCT-CACGATTTATAAGTGTTTT-CGTATATTAAACAA 657
C3N4F/R 569 bp  TAGATTTTGATATTAATGAAAATCTGCCCGATTTATAAGTGTTTTGCGCATATTAAACAA 536
                ************************ * ***************** ** ***********

BRUKX73TF       GGAAAAAATTTTT-AGTATTATTTTTTAGTGACAATTTTTAGTGTTATGAACATGTTGAG 716
C3N4F/R 569 bp  GGAAAAAATTTTTGAGTATTATTTTTTAGTGAG---------------------------- 569
                ************* ******************
```

Figure 18 Multiple alignment of nucleotide sequence of 569 bp from C3N4F/R primer amplification with *B. malayi* genome (BRUKX73TF).

```
CLUSTAL W multiple sequence alignment

C.elegans ced-3     ATCTGACGGGAAGGGTACGGCGAAATTATATTACCCAAACGCGAAATTTGCCATTTTGCG 4680
C3N4F/R 569 bp      ------------------------------------------------------ATTGAAGN 8
                                                                           ***

C.elegans ced-3     CCGAAAATGTGGCGCCCGGTCTCGACACGACAATTTGTGTTAAATGCAAAAATGTATAAT 4740
C3N4F/R 569 bp      TGTTAAATGACAACACCGG----GATATGGCCATTTCCCTTGGTTGCTAAA-TAATCAAT 63
                        *****      ****    ** * * * ****   **   *** *** *    ***

C.elegans ced-3     TTTGCAAAAAACAAAATTTTGAACTTCCGCGAAAATGATTTACCTAGTTTCGAA-ATTTT 4799
C3N4F/R 569 bp      TAATTTAAAAATATCTTTTT-----TTTAATTAAATGAATTGCTATTTTTCAATTGTGTT 118
                    *     ***** *   ****     *      ****** ** *    **** *   * **

C.elegans ced-3     CGTTTTTTCCGGCTACATTATGTGTTTTTTCTTAGTTTTTCTATAATATTTGATGTAAAA 4859
C3N4F/R 569 bp      AACAATTAATTAAAACAAAATTTGATAATACTAGAATTTATTTCCTAGTTTAATATACAA 178
                         **       ***  ** ** *  * **    ***  *      *** ** ** **
```

```
C.elegans ced-3     AACCGTTTGTAAATTTTCAGACAATTTTCCGCATACAAAACTTGA---TAGCACGAAATC 4916
C3N4F/R 569 bp      AAGCTCTTAAAAATTATAGGA--ATTTCATAAAATCAATACTTGAATTTGAAATAAAAAT 236
                    ** *  **  ***** *  **  ****     *  *** ******   *   *  ***

C.elegans ced-3     AATTTTCTGAATTTTCAAAATTATCCAAAA--ATGCACAATTTAAAATTTGTGAAAATTG 4974
C3N4F/R 569 bp      TAATTTTT--ATTTTAAAAATGATTTAAAACTATGAAAAATTAGAAATTTTATTAATTTC 294
                     * *** *  ***** ***** **  ****  *** * ****  ******    ** **

C.elegans ced-3     GCAAACGGTGTTTCAATATGAAATGTATTTTTAAAAACTTTAAAAACCACTCCGGAAAAG 5034
C3N4F/R 569 bp      GCTA-CTGAATTACTATTCAAAAGTTAATTACTAAAGTTTTTTTTATTATTTAAGATGTG 353
                    ** * * *  ** * **   ***  ** **   ***  ***    *  * *   **   *

C.elegans ced-3     CAATAAAAATCAAAACAACGTCACAATTCAAATTCAAAAGTTATTCATCCGATTTGTTTA 5094
C3N4F/R 569 bp      AAATG---ACCACAACA----CATAGGATAAATTCCTGCGTAACGCATATAAAATAATCA 406
                     ***    * ** ****    ** *    ******    ** *  ***   *  *  * *

C.elegans ced-3     TTTTTG-CAAAATTTGAAAAAATCATGAAGGATTTAGAAAAGTTTTATAACATTTTTTCT 5153
C3N4F/R 569 bp      ATTGAGTCCTAAATATTTATTTTTTTTAATGATTCATTTCTATATTTGAAATTTATTTTT 466
                     **  * *  ** *    *   *  * ** **** *      * **  **  ** *** *

C.elegans ced-3     AGATTTTTCAAAATTTTTTTTAACAAATCGAGAAAAAGAGAATGAAAAATCGATTTTAAA 5213
C3N4F/R 569 bp      ATGTCAAATTTAGATTTTGATATTAATGAAAATCTGCCCGATTTATAAGT--GTTTTGCG 524
                    *  *       *  ****  **  **    *        ** * * ** *   ****

C.elegans ced-3     AATATCCACAGCTTCGAGAGTTTGAAATTACAGTACTCCTTAAAGGCGCACACCCCATTT 5273
C3N4F/R 569 bp      CATATTAAACAAGGAAAAAATTTTTGAGTAT--TATTTTTTAGTGAG------------ 569
                     ****  *        * * ***   * **   ** *  ***  *
```

Figure 19 Nucleotide sequence comparision of 569 bp from C3N4F/R amplification to *ced-3* of *C. elegans*.

CLUSTAL W multiple sequence alignment

```
BRHDH13TF       ------------------------------------------------------------
C4N1F/R 474 bp  TCAACTGACGTGACATGCTCCCACCAGCCAGGGCGTGAAAGCGACCAGCTCACTTCCCGA 60


BRHDH13TF       -------------ATTTTTAGGTTAACATCTTCACATATACAAAGTTATTCTATGATTCA 47
C4N1F/R 474 bp  CATAAGTGTGATCTATTCTAGGTTAACATCTTCCCATATACAAAGTTATCCTATGATTCA 120
                            ** *************** *************** **********

BRHDH13TF       TTTCTCCACGACATATGTCGATGGTTGGTAATGGCACGATAATTTCTGGATGTGATCGTG 107
C4N1F/R 474 bp  TTTCTCCACGACTTATGTCGATGGTTGGTAATGGGACGATAATTTCTGGATGTGATCGTG 180
                ************ ******************** ************************

BRHDH13TF       TTTAAATGTTTTCAACCATGAAATTCTTCCTCTTATATAATTAATTAATTAAAGACTTTT 167
C4N1F/R 474 bp  TTCCAATGGTTTCAACCATGGAATTCTTCCTCTTATATAATTACTTAATTAAAGACTTTT 240
                **  **** *********** ********************** ****************

BRHDH13TF       CCACACGGATTATTTTCTCCATTTTATGCATTTTAAATTAGCGATCCTTTTTGCTTTGTT 227
C4N1F/R 474 bp  CCACACGGATTAATTTCTCCAGTTAATGCACTTTAAATTAGCGGTCCTTTCTGCCTTGTT 300
                ************ ******** ** ***** ************ ****** *** *****

BRHDH13TF       TTTCAACTCGAAACTTTCAATGATTTAATAATTTGCAGTCACATTATGCAATATTTATTC 287
C4N1F/R 474 bp  TTTCAACTCGAACCTTTCAATGAATTAATAATTTGCAGTCACCTTATGCAATATTTATTC 360
                ************ ********** ****************** *****************

BRHDH13TF       AGGCTAAAAAATTTTC-------------------------------------------- 303
C4N1F/R 474 bp  AGGCTGGAAAATTTTAACCCAGGGTTCACTTTAAAGTTTCCAATCACGACTTCCTCCTGA 420
                *****  ********

BRHDH13TF       ------------------------------------------------------
C4N1F/R 474 bp  GGGAGCTGGCCTAAAACCCTTACAGCTGAAGCATTCGATTTCGCAGAGCATAAT 474
```

Figure 20 Nucleotide sequence comparison of 474 bp from C4N1F/R amplification to *B. malayi* genome (BRHDH13TF).

Figure 21 Nucleotide sequence comparison of 474 bp from C4N1F/R amplification to *ced-4* of *C. elegans*

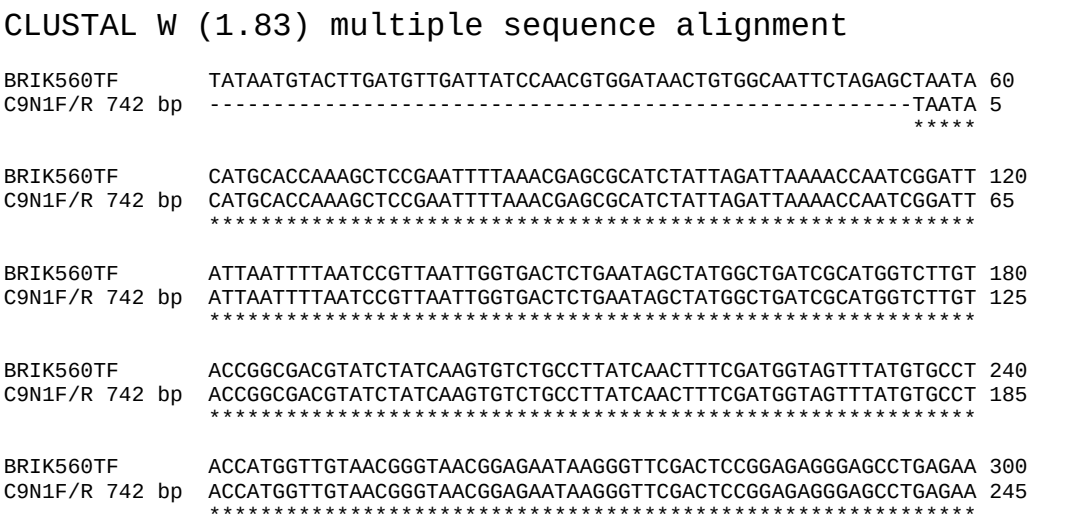


Figure 22 Nucleotide sequence comparison of 742 bp from C9N1F/R amplification to *brik-560* of *C. elegans*

```
BRIK560TF       ACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCACTCTCAGAATGAGGA 360
C9N1F/R 742 bp  ACGGCTACCACATCCAAGGAAGGCAGCAGGCGCACAAATTACCCACTCTCAGAATGAGGA 305
                ******************************** *************************

BRIK560TF       GGTAGTGACGAAAAATAACGAGACCGTTCTCTTTGAGGCCGGTTATCGGAATGGGTACAA 420
C9N1F/R 742 bp  GGTAGTGACGAAAAATAACGAGACCGTTCTCTTTGAGGCCGGTTATCGGAATGGGTACAA 365
                ************************************************************

BRIK560TF       TTTAAACCTGTTAACGAGGATCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAAT 480
C9N1F/R 742 bp  TTTAAACCTGTTAACGAGGATCTATGAGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAAT 425
                ************************************************************

BRIK560TF       TCCAGCTCTCAAAGTGTATATCGTCATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGC 540
C9N1F/R 742 bp  TCCAGCTCTCAAAGTGTATATCGTCATTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGC 485
                ************************************************************

BRIK560TF       GTCTCAGGACCTGGTCCATCCATTGGATGAGAACTAGGCTCCTAGGCTAATATTGCCAGT 600
C9N1F/R 742 bp  GTCTCAGGACCTGGTCCATCCATTGGATGAGAACTAGGCTCCTAGGCTAATATTGCCAGT 545
                ************************************************************

BRIK560TF       TTTCCCATACGTTACCTTAATTGGTTGCATATGGTGGCTAGCAAGTTTACCTTGA-AAAA 659
C9N1F/R 742 bp  TTTCCCATACGTTACCTTAATTGGTTGCATATGGTGGCTAGCAAGTTTACCTTGGGAAAA 605
                ************************************************  ****

BRIK560TF       ATTAGAGTGCTCAATGCGGGCTAATGCCTGAATACTCGTGCATGGAATAATGAAATAGGA 719
C9N1F/R 742 bp  ATTAGAGTGCTCAATGCGGGCTAATGCCTGAATACTCGTGCATGGAATAATGAAATAGGA 665
                ************************************************************

BRIK560TF       TCTCGGTTCTATTTTGTTGGTTTTCTGATCTGAGATAATGGTTAAGAGGGACGGACGGGG 779
C9N1F/R 742 bp  TCTCGGTTCTATTTNGTNGGTTT-CTGATCTGAGATA-TGGTTAA-AGGGACGGACGGGG 722
                ************** ** ***** ************* ******* **************

BRIK560TF       GCATTCGTATCGCTGCGTGAGAGGTGAAATTCTTGGACCGTAGCGAGACGTACGACTGCG 839
C9N1F/R 742 bp  GCATCCGTATCGCTGCGTGA---------------------------------------- 742
                **** ***************

BRIK560TF       AAAGCATTTGCCAAGAATGTCTTCATTAATCAAGAACGAAAGTCAGAGGTTCGAAGGCGA 899
C9N1F/R 742 bp  ------------------------------------------------------------


BRIK560TF       TCAGATACCGCCCTAGTTCTGACCGTAAACGATACCAACTAGCGTTCCGTCGGCGGTAAA 959
C9N1F/R 742 bp  ------------------------------------------------------------


BRIK560TF       TACGCCTTGACGGGCAG 976
C9N1F/R 742 bp  -----------------
```

Figure 22 Nucleotide sequence comparison of 742 bp from C9N1F/R amplification to *B. malayi* genome BRIK560TF.

CLUSTAL W multiple sequence alignment

```
C.elegans ced-9 ATGACACGCTGCACGGCGGACAACTCGCTGACGAATCCGGCGTATCGGCGACGAACGATG 60
C9N1F/R 742 bp  ------------------------------------TAATACATGC-ACCAAAGCTC 20
                                                     **   ** ** ** * *

C.elegans ced-9 GCGACTGGCGAGATGAAGGAGTTTCTGGGGATAAAAGGCACAGAGCCCACCGATTTTGGA 120
C9N1F/R 742 bp  CGAATT--TTAAACGAGCGCATCTATTAGATTAAAACCAATCGGATT-ATTAATTTTAAT 77
                   * *    * * **  *  * * *  *  *****   *  *     *   *****

C.elegans ced-9 ATCAATAGTGATGCTCAGGACTTGCCATCACCGAGTAGGCAGGCTTCGACGCGAAGAATG 180
C9N1F/R 742 bp  CCGTTAATTGGTGACTCTGAATAGCTATGGCTGATCGCATGGTCTTGTACCGGCGACGTA 137
                      * ** **     ** * ** **  * **       * ***  **  *     *

C.elegans ced-9 TCCATCGGAGAGTCAATTGATGGAAAAATCAATGATTGGGAAGAGCCAAGGCTTGATATC 240
C9N1F/R 742 bp  TCTATCA-AGTGTCTGCCTTATCAACTTTCGATGGTAGTTTATGTGCCTACCATGGTTGT 196
                ** ***  ** ***         **   ** *** * *   *    *    * ** *
```

```
C.elegans ced-9 GAGGGATTTGTGGTCGACTATTTCACGCACCGAATCCGGCAAAACGGAATGGAATGGTTT 300
C9N1F/R 742 bp  AACGG----GTAACGGAGAATA--AGGGTTCGACTCCGG--AGAGGGA--------GCCT 240
                 * **    **    **  **   * *   *** *****  * * ***        *  *

C.elegans ced-9 GGAGCACCGGGATTGC-CGTGTGGAGTGCAACCGGAGCACGAAATGATGCGAGTTATGGG 359
C9N1F/R 742 bp  GAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCACAAATT---ACCCACTCTCAG 297
                *    ** *  *   *      ***  *** * ** **** ** *    *    * *  *

C.elegans ced-9 AACGATATTCGAGAAGAAGCACGCGGAAAATTTTGAGACC---TTCTGTGAG-CAGCTGC 415
C9N1F/R 742 bp  AATGA------GGAGGTAGTG-ACGAAAAATAACGAGACCGTTCTCTTTGAGGCCGGTTA 350
                ** **       ** * **    ** *****   ******    *** **** * * *

C.elegans ced-9 TCGCAGTGCCCAGAATCTCATTTTCACTGTATCAGGATGTGGTTCGGACGGTTGGAAATG 475
C9N1F/R 742 bp  TCGGAATGGGTACAATTTAAACCTGTTAACGAGGATCTATGAGAGGGCAAGTCTGGTGCC 410
                *** * **   * *** * *   *             * **    **   **  *

C.elegans ced-9 CACAGACAGATCAATGTCCAATGTCTTATGGACGTTTGATAGGTCTAATCTCGTTCGGCG 535
C9N1F/R 742 bp  AGCAGCCGCGGTAAT-TCCAGC-TCTCA-AAGTGTAT-ATCGTCATTGCTGCGGTTAAAA 466
                  *** *     *** ****   *** *     ** * ** *   *     ** *

C.elegans ced-9 GTTTCGTAGCTGCAAAAATGATGGAATCCGTGGAACTGCAG--GGACAAGTGCGAAACCT 593
C9N1F/R 742 bp  AGCTCGTAGTTGGATCTGCGTCTCAGGACCTGGTCCATCCATTGGATGAGAACTAGGCTC 526
                   ****** ** *     *    *   * ***  *  *    ***  **  * *  *

C.elegans ced-9 CTTCGTTTACACATCGCTGTTCATCAAAACGCGGATCCGCAACAACTGGAAGGAACACAA 653
C9N1F/R 742 bp  CTAGGCTAATATTGC-CAGTTTTCCCATACGT-----TACCTTAATTGGTTGCA----TA 576
                **  * * * *   * * ***   * * ***        *   ** ***  * *     *

C.elegans ced-9 TCGGAGCTGGGACGACTTCATGACACTCGGAAAACAAATGAAAGAGGACTACGAACGAGC 713
C9N1F/R 742 bp  TGGTGGCTAG--CAAGTTTAC----CTTGGGAAA--AATTAGAGTGCTCAATG--CGGGC 626
                * *  *** *  * * ** *     ** ** ***  *** * ** *  * * *  ** **

C.elegans ced-9 AGAAGCTGAAAAAGTGGGACGCCGGA-AGCAGAACAGA----CGGTGGTCGATGATTGGC 768
C9N1F/R 742 bp  TAATGCCTGAATACTCGTGCATGGAATAATGAAATAGGATCTCGGTTCTATTTNGTNGGT 686
                  * **   ** * * *  *   * * *    ** **     ****  *   *  * **

C.elegans ced-9 GCTGGAGTAACAGCTGGAGCCATTGGAATCGTTGGAGTCGTCGTGTGTGGGCGGATGATG 828
C9N1F/R 742 bp  TTCTGA-TCTGAGATATGGTTAAAGGGACGGACGGGGGCATC-CGTATCGCTGCGTGA-- 742
                    ** *   ** *   *  *  ** *  *  ** * * **  ** * *  *  ***

C.elegans ced-9 TTCAGCTTGAAGTAA 843
C9N1F/R 742 bp  ---------------
```

Figure 23 Nucleotide sequence comparison of 742 bp from C9N1F/R amplication to *ced-9* of *C. elegans*.

6. ORF Finder (Open Reading Frame Finder)

The nucleotide sequence of putative *ced-3*, *ced-4* and *ced-9* gene of *B. malayi* were analyzed for their open reading frame by using ORF finder from BLAST program. The results are shown in Figure 24-26. In Figure 24, there was no ORF found in the nucleotide sequence from C3N4F/R amplification. There are two and three open reading frames in the sequences of C4N1F/R (474bp) and C9N1F/R amplification (Figure 25 and 26).

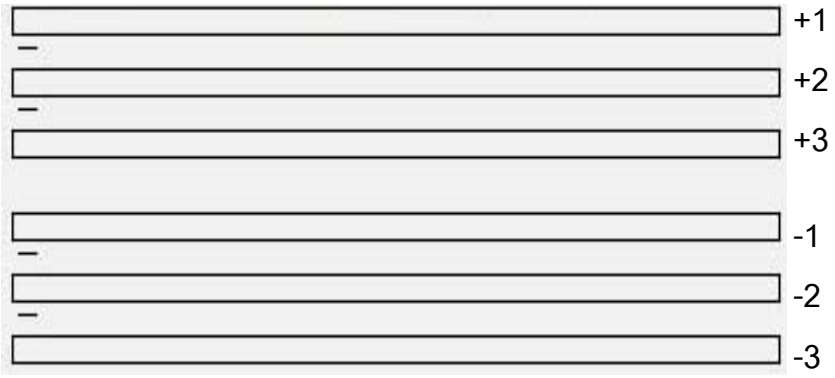
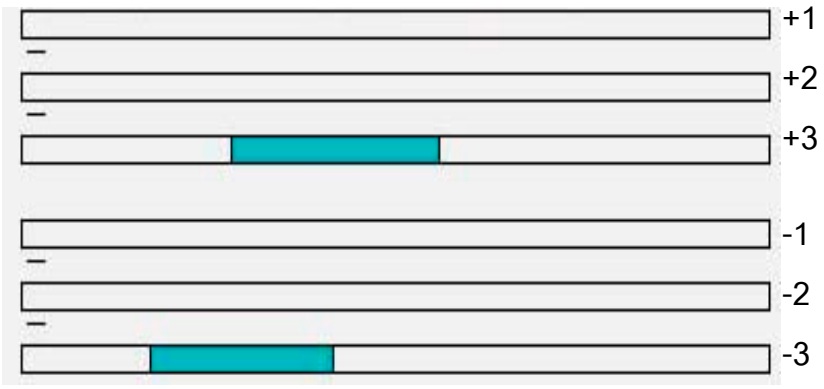


Figure 24 Open reading frame (ORF) analysis of nucleotide sequence from C3N4F/R amplification.



Frame	form to	Length
+3	135-266	132
-3	83-199	177

Figure 25 Open reading frame (ORF) analysis of nucleotide sequence from C4N1F/R amplification.

Frame	form to	Length
-2	376-504	129
-2	616-726	111
+2	104-205	102

Figure 26 Open reading frame (ORF) analysis of nucleotide sequence from C9N1F/R amplification.

7. Comparison of amino acid sequences

Amino acid sequence of ORF from putative *ced-4* and *ced-9* genes of *B. malayi* were analyzed for their similarity and identity with apoptosis protein of *C. elegans* by using Clustal W program. The multiple alignments of each amino acid sequence are shown in Figure 27-31.

```
CLUSTAL W (1.83) multiple sequence alignment

CED4_C.elegans  LGNVPKQMTCYIREYHVDRVIKKLDEMCDLDSFFLFLHGRAGSGKSVIASQALSKSDQLI 180
Ced_4_ORF+3     ------------------------------------MSMVGNG-TIIS---------- 11
                                                    . .*.* ::*:

CED4_C.elegans  GINYDSIVWLKDSGTAPKSTFDLFTDILLMLARVVSDTDDSHSITDFINRVLSRSEDDLL 240
Ced_4_ORF+3     GC--DRVPMVS--------TMEFF----LLYN---------YLIKDFSTR---------I 39
                *   * :  :.        *:::*    *:           : *.** .*         :

CED4_C.elegans  NFPSVEHVTSVVLKRMICNALIDRPNTLFVFDDVVQEETIRWAQELRLRCLVTTRDVEIS 300
Ced_4_ORF+3     NFSS------------------------------------------------------- 43
                **.*
```

Figure 27 Amino acid sequence alignment of ORF from *ced-4* frame +3 compared to CED-4 protein of *C. elegans*

```
CLUSTAL W (1.83) multiple sequence alignment

CED4_C.elegans  MLCEIECRALSTAHTRLIHDFEPRDALTYLEGKNIFTEDHSELISKMSTRLERIANFLRI 60
Ced_4_ORF-3     ------------------------------------------------------------


CED4_C.elegans  YRRQASELGPLIDFFNYNNQSHLADFLEDYIDFAINEPDLLRPVVIAPQFSRQMLDRKLL 120
Ced_4_ORF-3     -------------------------MVETIG-TRSHPEIIVPL---PTID---ISR--- 24
                                         : : *. : ..*::: *:   * :.   :.*

CED4_C.elegans  LGNVPKQMTCYIREYHVDRVIKKLDEMCDLDSFFLFLHGRAGSGKSVIASQALSKSDQLI 180
Ced_4_ORF-3     -GEMNHRITLYMGR------------C------------------------------- 38
                 *:: :::* *: .             *

CED4_C.elegans  GINYDSIVWLKDSGTAPKSTFDLFTDILLMLARVVSDTDDSHSITDFINRVLSRSEDDLL 240
Ced_4_ORF-3     ------------------------------------------------------------
```

Figure 28 Amino acid sequence alignment of ORF from *ced-4* frame -3 compared CED-4 protein of *C. elegans*

```
CLUSTAL W (1.83) multiple sequence alignment

CED-9_C.elegans        TIFEKKHAENFETFCEQLLAVPRISFSLYQDVVRTVGNAQTDQCPMSYGRLIGLISFGGF 180
Ced_9_ORF+2            ------------MADRMVLYRR---RIYQ--VSALSTFDGSLCAY-HG-------CNG- 33
                                  :.::::   *    :**  * ::.. : . *.  :*        .*

CED-9_C.elegans        VAAKMMESVELQGQVRNLFVYTSLFIKTRIRNNWKEHNRSWDDFMTLGKQMKEDYERAEA 240
Ced_9_ORF+2            ------------------------------------------------------------
```

Figure 29 Amino acid sequence alignment of ORF from *ced-9* frame +2 compared to CED-9 protein of *C. elegans*

```
CLUSTAL W (1.83) multiple sequence alignment

CED-9_C.elegans        SIGESIDGKINDWEEPRLDIEGFVVDYFTHRIRQNGMEWFGAPGLPCGVQPEHEMMRVMG 120
Ced_9_ORF-2.1          ------------------------------------------------------------MD 2
                                                                                   *.

CED-9_C.elegans        TIFEKKHAENFETFCEQLLAVPRISFSLYQDVVRTVGNAQTDQCPMSYGRLIGLISFGGF 180
Ced_9_ORF-2.1          QVLRRR--SNYELFNR---SNDDIHFESWN--YR--G--WHQTCPLIDPR---------- 41
                        ::.::  .*:* * .   :   * *. ::   *  *    : **:   *

CED-9_C.elegans        VAAKMMESVELQGQVRNLFVYTSLFIKTRIRNNWKEHNRSWDDFMTLGKQMKEDYERAEA 240
Ced_9_ORF-2.1          ------------------------------------------------------------
```

Figure 30 Amino acid sequence alignment of ORF from *ced-9* frame -2 (376-504) compared to CED-9 protein of *C. elegans*

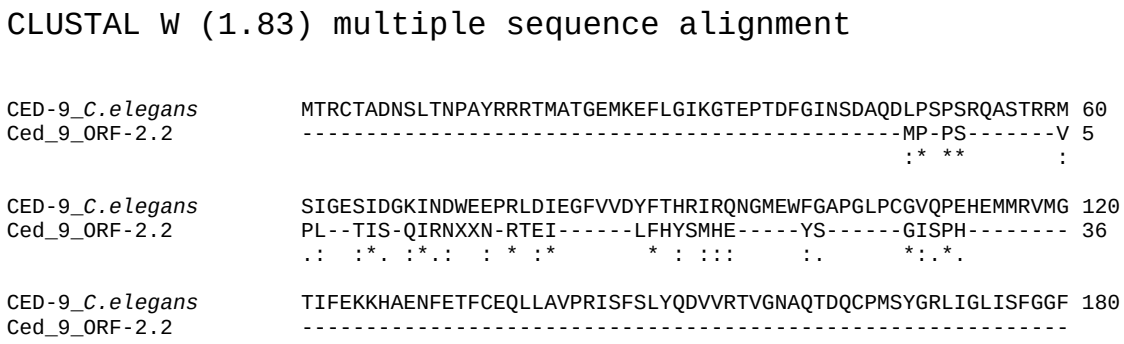


Figure 31 Amino acid sequence alignment of ORF from *ced-9* frame -2 (616-726) compared to CED-9 protein of *C. elegans*

CHAPTER 5

DISCUSSION

Lymphatic filariasis, caused by *B. malayi* and *W. bancrofti* nematode parasites, is one of the target organisms of the WHO for which special research programs exist, with the ultimate aim being to find new treatments.

In multi-cellular organisms, cell growth and development can be controlled by apoptosis which is defined by a sequence of regulated events. Apoptosis is an essential part of cell biology and is thought to have evolved not only to regulate growth and development in multi-cellular organisms but also to guard against viral, bacterial, parasitic infections.

In this study, the putative *ced –3, ced–4* and *ced–9* genes that might be regulated apoptosis process in *B. malayi* were cloned, sequenced and analyzed for nucleotide sequence homology.

Specimens

In this study, microfilaria sample collected from cat blood was used for DNA preparation. Cat is one of the hosts of *B. Malayi* which can be fed in laboratory and the blood sample can be withdrew many times (once per month). Even the blood volume from cat was low in each time but it is easier than taking the blood from patient who stay far away (in the Southern part) which can be taken blood only once.

For studying apoptotic genes in *B. malayi*, lower amount of blood sample makes the DNA the best candidate comparing to mRNA or proteins. It is easier to amplify the interested genes from little amount of DNA than the purification of mRNA that makes up only 1-5% of total cellular RNA. For protein studying, it needs a large amount of parasite lysate which is very difficult to get from blood cat.

If analyze by ELISA methods, the proteins need partially characterize to inform about antibody need to detect the protein. But no information about the apoptotic protein in *B. malayi* and knowledge about the relation of these proteins in Filaria with the other parasite have been reported. Therefore, proteins are hardly used for studying apoptosis process in *B. malayi*.

***C. elegans* was used as a model for studying of programmed cell death in filarial**

Apoptosis programmed cell death is a conserved, gene–directed mechanism for elimination of unnecessary or dangerous cells from organism. Although, the Filarial Genome Project is already been set up, but it is still on the process of studying. In addition, apoptosis genes in filarial parasite have not been reported.

The core cell death pathway was first defined through genetic analysis in the nematode *C. elegans*.[84] This worm has been extensively used as a model system for studying of basic biological processes.[85] By comparison, the *C. elegans* genome has several similarities to *B. malayi*, for examples, it has the same genome size (100 Mb) which organized as five chromosomes.[86-87]

The EST sequences generated by the FGP can now be used in genome-wide comparison with the *C. elegans* data set. Most of the genes in the *Brugia* EST shaves as their closet homologue to *C. elegans* genes.[88] The evolutionary distance between *C. elegans* and *Brugia* is great and, thus, any shared sequence motifs are likely to be functionally important. The closeness of the similarities will suggest whether it makes sense to examine the biology of system well understood in *C. elegans* and other important parasites.[85]

Therefore, in this study, the nematode *C. elegans* was used as a model system for studying apoptotic genes in *B. malayi*.

Isolation of apoptotic genes from *B. malayi* genomic DNA

A putative *ced-3* gene of *B. malayi* was isolated using 3 sets of primer designed from *ced-3* gene of *C. elegans*. The nucleotide sequence from PCR product of C3N4F/ C3N4R amplification was aligned with the data in the GenBank. The result revealed that it was similar to *ced-3* gene of *C. elegans* with 52% identity and also showed 96% identity to BRUKX73TF gene of *B. malayi* genome (Filaria genome project) but this gene has not identified yet.

The nucleotide sequence of putative *ced-4* gene of *B. malayi* was compared with nucleotide sequence of *ced-4* gene from *C. elegans* and the genes in *B. malayi* genome. The result from C4N1F/R amplification showed the highest identity with *ced-4* gene of *C. elegans* at 53% and 92% identity with BRHDH13TF gene of *B. malayi*. Therefore, two sets of inner sequences were designed from 474 bp and 591 bp DNA fragments (the PCR products from C4N1F/R amplification) and re-amplified for more specific gene product.

Unfortunately, no amplification product was obtained at optimized temperature. These may be the result of none-specific of the primers with the target gene.

The nucleotide sequence of PCR products from *ced-9* primers (C9N1F/R) were compared with nucleotide sequence of *ced-9* gene from *C. elegans* and the gene of *B. malayi*. The result revealed the highest identity with *ced-9* gene of *C. elegans* and BRIK560 gene from *B. malayi* at 46 % 98 %, respectively. Surprisingly, when the BRIK560TR gene was aligned with the data in GenBank, it was similar to small subunit ribosomal RNA (rRNA) gene of *B. malayi*.

Because the nucleotide sequence of PCR products showed homology to apoptosis genes of *C. elegans ced-3, ced-4*, and also showed similarity with *Brugia* gene with more than 90%. Thus, it can be concluded that the amplified products were the genes of *B. malayi* and may be assumed as apoptosis genes of *B. malayi*. However, a little is known about the structure of the apoptosis genes (such as exon and intron) and molecular mechanisms of apoptosis process in *B. malayi*. Therefore, more extensive studies are needed to fully characterize the apoptotic pathway and to define the factors that control apoptosis in the *Brugia* parasite.

For the studying of apoptosis genes in *B. malayi*, primers were designed from *C. elegans* which is the highest related organism to *B. malayi*. But no information about the homology of the apoptosis genes between these organisms, therefore, the annealing temperature of PCR reaction was decreased for promoting primer annealing which resulting in non-specific PCR products.

The searching for open reading frame (ORF) from nucleotide sequences by using ORF finder of BLAST program revealed that no ORF region presence in the sequence of C3N4F/R amplification, thus, it may be conclude that it was intron of the gene. The analysis of ORF from sequences of C4N1F/R and C9N1F/R amplification indicate that they were the exon region of the gene. However, the homology analysis by using amino acid sequence (Blastp program) revealed that there was no similarity with other gene in GenBank. This sequence then possibly a new gene, which is very interesting for future study.

The understanding of the apoptotic pathway could provide information regarding their pathogenesis, which could be exploited to target new drugs to limit their growth and treatment for filariasis.

For the future study, it needs to determine the full-length of apoptotic genes, ability of gene, gene function and correlation with other genes.

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APPENDICES

APPENDICES

Reagent

1. Phosphate buffered saline (PBS buffer)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
H_2O (distilled water)	800 ml
Adjust pH 7.4 with HCl	
H_2O (distilled water) adjust volume to	1000 ml

2. LB broth

Bacto™ peptone	10 g
Yeast extract	5 g
NaCl	10 g
H_2O (distilled water) adjust volume to	1000 ml

3. LB-ampicillin agar plate

Bacto™ peptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g
H_2O (distilled water)	1000 ml
Ampicillin 100 μg/ml	

4. IPTG 200 mg/ml

IPTG (Isopropyl-β-D-thiogalactopyranoside, dioxane free)	1 g
H_2O (distilled water) (sterile)	5 ml

Reagent

5. X-Gal 20 mg/ml

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)	100 mg
N, N-dimethylformamide	5 ml

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